Epidemiology of brucellosis among humans and animals in Goa region

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By

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Statement

As required under the University ordinance, I hereby state that the present thesis for Ph.D. degree entitled "**Epidemiology of brucellosis among humans and animals in Goa region**" is my original contribution and that the thesis and any part of it has not been previously submitted for the award of any degree/diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area. The literature related to the problem investigated has been cited. Due acknowledgement have been made whenever facilities and suggestions have been availed of.

As suggested by the External Examiners, appropriate corrections are incorporated in the thesis in relevant pages.

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Certificate

Certified that the research work embodied in this thesis entitled "**Epidemiology of brucellosis among humans and animals in Goa region"** submitted by **Mr. Ajay Dilip Pathak** for the award of **Doctor of Philosophy** degree in **Microbiology** at Goa University, Goa, is the original work carried out by the candidate himself under my supervision and guidance.

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Abbreviations/Acronyms

Abbreviation	Full form	Abbreviation	Full form
%	Per cent	CFT	Complement Fixation Test
ELISA	Enzyme linked immunosorbent assay	BPAT	Buffered plate agglutination test
cELISA	Competitive Enzyme-linked immunosorbent assay	MRT	Milk Ring Test
AB-ELISA	Avidin-biotin ELISA	2- MET	2- Mercaptoethanol Agglutination Test
iELISA	Indirect Enzyme linked immunosorbent assay	RBPT	Rose Bengal Plate Test
dot- ELISA	Dot Enzyme-linked immunosorbent assay	STAT	Standard Tube Agglutination Test
IgG	Immunoglobulin G	FPA	Fluorescence Polarization Assay
IgM	Immunoglobulin M	dNTPs	Deoxynucleotide triphosphates
e.g	Example	SARS	Severe Acute Respiratoy Syndrome
et al.	et alii	MZN	Modified Ziehl-Neelsen
etc.	et cet·er·a	PFGE	Pulsed Field Gel Electrophoresis
IS	Insertion sequence	RFLP	Restriction fragment length polymorphism
I.U.	International Unit	OIE	Office International des Epizooties
EDTA	Ethylene diamine tetra acetic acid	CFSPH	Centre for fod security and public health
LPS	Lipopolysaccharide	WHO	World Health Organisation
OPS	O- Polysaccharide	FAO	Food and Agriculture Organization USA
bcsp	*Brucella* cell surface protein	MLST	Multiple locus sequence typing
TLR	Toll like receptor	MLVA	Multiple locus variable number tandem repeats analysis
USDA	United States Department of Agriculture	PUO	Pyrexia of unknown origin

Units of measurement

μg: microgram

μm: micrometer

g: gravitational force

gm: grams

h: hour

kDa: Kilodaltons

M: Molar

mg: mili grams

ml: milliliter

mM: mili Molar

mm: millimeter

ng: nanogram

O.D.: Optical density

^{o}C: Degree Celsius

Pmol: Pico mole

rpm: revolution per minute

μ: micron

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Chapter 1

General Introduction

Epidemiology involves study of distribution and determinants of the disease in a population. Diseases can be classified based on nature of causative agents. Majority of devastating infections affecting human and animals are zoonotic, which are naturally transmissible between animals and humans (WHO, 1959). Zoonoses are leading causes of human morbidity and mortality. Investigations of the impact of zoonoses in many countries are lacking and their burden in human populations has not been estimated accurately.

As per latest information, zoonotic disease constitutes 58% of the 1407 recognized species of human pathogens (Woolhouse and Gowtage- Sequeria, 2005). Currently, avian influenza, Bovine Spongiform Encephalopathy (BSE), Hantavirus, SARS associated coronavirus and the Nipah virus are considered as important emerging pathogens. While rabies, bovine brucellosis, yellow fever and bovine tuberculosis are re-emerging in many parts of world affecting lives of people and animals.

Similarly, food borne zoonoses are major causes of mortality and morbidity among humans and animals across the world. Some zoonotic infections also affect food-producing animals resulting in reduced availability of animal-derived foods and reduced wealth to farmers. Brucellosis is caused by the zoonotic pathogen, *Brucella* spp. It has significant effect on public health. Brucellosis, an re-emerging disease, is known by various names including Mediterranean fever, undulant fever, Malta fever, enzootic abortion, epizootic abortion, contagious abortion and Bang's disease (Cutler et al., 2005; Pappas et al., 2006). It is a highly contagious bacterial infection which affects general well being in humans and reduced fertility among animals (Corbel, 1997). Sporadic outbreaks among livestock and human population have been reported throughout the world. Brucellosis in pregnant animals is characterized by abortions in

last trimester of pregnancy, infertility and other reproductive problems in animals (Nicoletti, 2010). Human brucellosis occurs as febrile illness leading to chronic debilitating complications (Seleem et al., 2010; Franco et al., 2007). The disease can cause substantial disabilities but has low fatality rates. The members of genus *Brucella* are Gram-negative, non-capsulated, non spore-forming coccobacilli (Seelam et al., 2010, Godfroid et al., 2005; Gwida et al., 2010). *Brucella* species have high levels of genetic similarity; however, differ broadly in host tropisms and pathogenicity.

Brucella spp. belongs to the class α-proteobacteria having close genetic relationship with plant pathogens belonging to the genera *Agrobacterium* and *Rhizobium;* animal pathogen- *Bartonella* and soil bacteria of the genus *Ochrobactrum*. Taxonomically, genus *Brucella* belongs to Order- Rhizobiales, Family- *Brucellaceae* and currently comprised of 10 species affecting distinct primary hosts. Earlier, the genus consisted of six classically recognized species (or nomen species) classified on the basis of antigenic/biochemical characteristics and primary host specificity. *B. abortus* primarily affects cattleand buffaloes, *B. melitensis* affects sheep and goats and *B. suis* primarily affecting swine, *B. ovis* causes ram epididymitis in sheep, whereas, *B. canis* affects dogs and *B. neotomae* was isolated from desert wood rats. Except *B. ovis* and *B. neotomae,* all other species are of zoonotic importance.

Brucella ceti and *Brucella pinnipedialis* were isolated from marine mammal hosts (whales, dolphins, porpoises and seals) (Foster et al., 2007). *B. microti* was isolated from the common vole and later from the red fox, and soil (Scholz et al., 2008a; Scholz et al., 2008b; Scholz et al., 2009). *B. inopinata* was isolated from a patient with human breast implant (Scholz et al., 2010). Strain BO2 isolated from a

case of chronic destructive pneumonia (Tiller et al., 2010b) and strains isolated from wild Australian rodents form a novel lineage of the *B. inopinata* species (Tiller et al., 2010a; Tiller et al., 2010b). A novel *Brucella* species has been isolated from African bull frogs (Eisenberg et al., 2012). Similarly, a novel species, *B. papionis* sp. nov was isolated from clinical specimens of baboon- *Papio* spp. (Wharmore et al., 2014).

Brucella species can be biotyped using their cultural and serological properties (Alton et al., 1975). Currently, *B. abortus* has eight biotypes (1-7 and 9), *B. melitensis* has three (1-3), and five (1-5) biotypes are known for *B. suis* (Osterman and Moriyon, 2006). The genomes of the species within genus *Brucella* are distinctive. Two distinct chromosomes are present in *B. abortus, B. melitensis,* and some biotypes of *B. suis,* while, another *B. suis* biotype contains a single larger chromosome (Pappas et al., 2005).

Brucellosis is an ancient disease. Genome of the bacterium *B. melitensis* has been recovered during exploration of ruins of a Medieval Italian village from a 700-year-old skeleton using shotgun metagenomics (Kay et al., 2014). Similarly, a more than 2-million-year-old male skeleton of *Australopithecus africanus* had vertebral lesions containing *Brucella* (D'Anastasio et al., 2009). Bronze Age skeletons from the Levant and the Basque country, adult skeletons from Herculaneum, and remains from medieval human were also found to have lesions consistent with brucellosis (D'Anastasio et al., 2011). Bone lesions showing brucellosis like features were observed in skeletal remains of people killed during the eruption of Mount Vesuvius in Rome (Capasso, 2002).

Historically, the disease was recognized in the Mediterranean region, particularly in goats and sheep. Britain maintained a military base on Island of Malta during the 18th and 19th centuries. During this period, several British physicians

provided vivid descriptions of illness in garrisoned troops and physician David Bruce was dispatched to investigate; he isolated the causative organism from four fatal cases in 1887 and named it *Micrococcus melitensis*. In 1897, Wright and Semple successfully used serum agglutination method for differentiating brucellosis from other febrile illnesses. Subsequently, the Commission of Mediterranean Fever was established in 1904 (Evans and Brachman, 2009). A Greek physician working with Bruce, Themistokles Zammit demonstrated in 1905 that the Maltese goat with no clinical signs of illness carried the organism and served as the source of infection through consumption of unpasteurized milk by military personnel. Therefore, goat's milk was banned and the troubling episode ended in 1906.

Concurrently in 1897, Bernard Bang, a physician-veterinarian, studied a disease of cattle in Denmark referred to as "contagious abortion" and isolated an organism that he named *Bacillus abortus*. Later an American microbiologist, Alice Evans showed in 1918 that it caused human brucellosis and was closely related both morphologically and biochemically to Bruce's organism. Karl Meyer, a veterinary scientist at the Hooper Foundation in San Francisco, proposed to group these organisms under the genus "*Brucella*" in honor of Dr. Bruce to settle the nomenclature issues. Alice Evans continued to develop improved techniques of recovering the organism and diagnosing the disease resulting in becoming infected herself in 1925 incidental to laboratory exposure. During this period "Bang's Disease" became the standard term for livestock; human disease was also termed Bang's as well as "Malta or Undulant Fever" for many years.

Brucella melitensis and *B. suis* have been considered as remarkable pathogens in biological warfare owing to their biological and pathological properties. At a dose of approximately 10-100 cells, brucellae are highly infectious through aerosol route.

As defined by Center for Disease Control and Prevention (CDC) *Brucella* spp. are classified as category B bioterrorism agents.

Although the disease has been reported worldwide, it has been brought under control in developed countries. Eradication of brucellosis was possible through test and slaughter programmes coupled with the vaccination of livestock. However, the spread from wildlife to domstic animals and the worldwide trade of animals promoted the global circulation of this disease (Pappas et al., 2006; Godfroid, 2002). Hence, it remained a major public health concern in many developed countries despite its control. Bovine brucellosis has been eradicated from many countries including Australia, North American and European countries. The disease is endemic in the Asia, African and Mediterranean countries, Central and South America and the Caribbean (Corbel, 1997). Incidence remains high in the near and Middle East and North African countries; it has been greatly reduced in Latin America (excluding Mexico and parts of Peru) and south Europe. Two new hyper endemic foci have emerged: the former Soviet republics of Asia, and the Balkans. Similarly, emergence of new focal points of brucellosis in humans has been reported in central Asia, with rapid worsening of the circumstances in few countries of the Middle East (Pappas et al., 2006). The absence of data from endemic areas such as India, China and sub-Saharan Africa, and also the possibility of rapid and diverse introduction of the disease in the non-endemic developed world, highlights the need for proper epidemiological surveillance at a global level.

In developing countries milk is the major source of protein along with meat and eggs. Animals including cattle, buffalo, sheep, goat and pigs are mainly reared for milk and meat production, and also they provide different by-products of commercial importance (Cutler et al., 2005). Animals get infected by different bacterial, viral or

parasitic organisms if proper precautions are not taken. Infection of livestock by *Brucella* spp. can lead to severe consequences involving abortion, endometritis, reduced fertility which affects productivity. Infection by *Brucella* results in heavy economic losses in animals due to loss of calves, wool, meat and milk production. In developing countries, 12% of animals have recent or current infections with brucellosis, reducing production by 8% (Grace et al., 2012).

Brucella species have been isolated from wild animals including bison, elk, feral swine, wild boar, fox, African buffalo, reindeer, and caribou (Godfroid, 2002). Recently, infections among seals, sea lions, walruses, dolphins, porpoises, whales and an otter have been reported. Contaminated housing and pastures through aborted material or secretions of infected carriers contribute to the dissemination of brucellosis among animal herds (Bercovich et al., 1998). Licking of fetuses that have been aborted and discharges from genital organs of an infected animal may transmit the disease orally. Feeding of contaminated colostrum and milk may also transmit the infection. Other routes of transmission include respiratory exposure, conjunctiva and damaged skin or mucosal membranes. Similarly congenital or perinatal infections have also been documented. Under natural conditions, venereal transmission is not a major route of infection; however, artificial insemination with contaminated semen is a potential source of infection (Carvalho Neta et al., 2010).

Zoonotic nature of *Brucella* spp. often results in infections among humans. The infection to humans generally occurs accidently from infected animals. The disease causes significant morbidity and can lead to severe debilitating complications which affect general well being of person. Thus, developing countries should undertake measures to control this pathogen by surveillance and eradication programs. For this, knowing the epidemiology of the disease in that region is

particularly important. Understanding the prevalence of disease in livestock is necessary to avoid outbreak situation in human and animal populations.

Humans get infected mainly by *B. abortus*, *B. melitensis* and *B. suis*, *B. canis* and species isolated from marine mammals affect humans rarely. Every year more than 0.5 million new cases of brucellosis are reported making it one of the most commonly encountered zoonosis worldwide (Pappas et al., 2006). The annual incidence rates vary from <2 to >500 per 1,000,000 population among various regions (Pappas et al., 2006). Prevalence rates in some countries exceeds ten cases per 1, 00,000 population (Mantur and Amarnath, 2008). Brucellosis has been documented as an occupational disease in endemic areas. People occupied in the livestock industry such asshepherds, farmers, abattoir associated personnels and veterinarians are frequently affected. Persons handling animal parturition are at a greater risk of infection (Cooper, 1992). Also, brucellosis is an important reason of travel-associated morbidity (Memish and Balkhy, 2004).

Human brucellosis is usually characterized by an acute or sub-acute febrile illness. It needs to be differentiated from many other infectious and non-infectious ailments. Patients with brucellosis usually present with an influenza-like or septicemic illness. The major manifestations of brucellosis in humans include undulating fever, loss of weight, night sweating, chills, headaches, low back ache, depression, muscle and joint pain (Dossey, 2010).

Infected animals may infect humans directly or indirectly thorugh occupaltionl or foodborne route. Close contact with animals may result in direct transmission through respiratory, conjunctival, and cutaneous routes. Consumption of unpasteurized or raw dairy products may transmit the infection indirectly (Young, 1995). Laboratory personnel handling the live bacterial cultures are at higher risk via

aerosol transmission. *Brucella* infections in humans and animals are usually under diagnosed or misdiagnosed due to non specific clinical presentation (Smits and Kadri, 2005). The disease in humans has protean manifestations which mimic other flu like infections. Brucellosis in animals is difficult to diagnose in absence of significant symptoms except abortion in pregnant animals and orchitis in males. Determination of prevalence in specified population is necessary to find out status of infection. Prevalence determination is based on accurate diagnosis of disease.

Isolation of the organism from blood cultures and clinical specimens, or detection of specific antibodies by serological tests is required for the diagnosis of brucellosis. Presumptive evidence of infection can be obtained by serological testing of the samples. Currently, no diagnostic test has been considered sufficiently sensitive and specific to detect all stages of infection in live animals (McGiven et al., 2003) or appropriate for all epidemiological situations (Godfroid et al., 2002; Nielsen et al., 2002). Rose Bengal Plate Test (RBPT), complement fixation tests (CFT), Coombs test and the ELISA are commonly used for diagnosis of bovine brucellosis (Nielsen, 2002; Abernethy et al., 2012). For screening herds and individual animals, RBPT and the buffered plate agglutination test, CFT, ELISA and fluorescence polarization assay (FPA) are suitable (OIE, 2009; Gall et al., 2004), while, RBPT and CFT are agreed for the screening of small ruminants (OIE, 2010). Diagnosis of brucellosis among humans involves use of RBPT, CFT, Coombs test, ELISA and Standard tube agglutination test (STAT) (Rubio et al., 2001; Mantur et al., 2010). Nowadays, the Coombs test is rarely performed in routine clinical laboratories, because the procedure is too complex, time consuming, and labor intensive, necessitating skilled personnel. Enzyme-linked immunosorbent assay (ELISA) can

readily identify individual IgM and IgG antibodies to the surface antigen of *B. abortus* and allows better correlation with the clinical condition.

Bacteriological isolation of the pathogen is the "gold standard" for the brucellosis diagnosis. The pathogen can be demonstrated by staining the smears prepared from clinical material such as placental bits, fetal stomach contents, deep vaginal swabs and semen (Alton et al., 1975).

Various standard culture media are used for isolation of brucellae from clinical samples including aborted fetuses, genital secretions, colostrum, milk, and synovial fluid collected from cases of joint infections. Similarly, lymph nodes (genital and oropharyngeal), spleen, and mammary gland and associated lymph nodes are suitable for isolation from slaughtered animals. Bacteriological diagnosis of brucellosis has to be done in specially equipped biosafety level 3 laboratories. Moreover, it is a very labor-intensive and time-consuming procedure. However, the isolation and cultivation of bacteria are also necessary preliminary steps for staining and biotyping of *Brucella* species. Biotyping of *Brucella* species, isolated from the biological samples, provides significant epidemiological data that allow tracing the focus of infection and the ways of its spread (Smirnova et al., 2013).

The DNA based methods like polymerase chain reaction (PCR) are rapid and reliable for diagnosis of brucellosis. Limitations of conventional methodology can be avoided using these techniques (Baddour, 2012). Several genus-specific PCR targeting the 16S rRNA and genes of different outer membrane proteins have been developed (Zerva et al., 2001; Queipo-Ortuρo et al., 2005). These techniques can be used to detect the DNA of the organisms from clinical samples. PCR methods can also be used for identification of some *Brucella abortus* biovars (Leal-Klevezas et al., 2000) and distinguishing between S19 and RB51 strains of *Brucella abortus* (Sangari

and Agüero, 1994; Vemulapalli et al., 1999). Various *Brucella* species including biovars can be identified using multiplex PCR assays. Genus *Brucella* demonstrated high degree of homology (>90%) through DNA–DNA hybridization (Verger et al., 1985), but still, molecular subtyping methods had proved to be useful for tracing *Brucella* infections.

In industrialized nations, brucellosis has been controlled through rigourous screening of domestic livestock and regular animal vaccination programmes (Corbel, 1997). Brucellosis is common among countries which lack standardized and effective public health and domestic animal health programmes. Complexity of conditions in various countries creates considerable difficulty to identify the risk factors and in turn to eradicate it (Pappas et al., 2006). Unrestricted trade, frequent movement of animals without testing, use of semen from unscreened infected bulls for artificial insemination and poor hygiene at farms are possibly attributed to the spread of the infection. In addition, increasing demand for foods of animal origin, and agricultural intensification has also caused high prevalence of disease.

India is an agrarian country. Rural economy is primarily based on agriculture and allied occupations including animal husbandry. Animal husbandry sector forms the large part of national economy. Animal species, cattle, buffalo, camel, yak, mithun, pig, sheep and goat are commonly reared in different regions of country based on adaptability of animals to environmental conditions and availability of feed. Dairy farming is a major source of income in India. Cattle and buffaloes are usually reared for milk production. Though, the productivity in dairy sector is increasing significantly, it is heavily reduced by diseases in animals. Brucellosis is mainly responsible for loss of profitability among dairy sector of India.

In India, presence of brucellosis has been proved epidemiologically in various species of domestic animals including cattle, sheep, goats, buffaloes, yaks, camel, horses and pigs. Seroprevalence reports highlights that bovine brucellosis is endemic in India (Renukaradhya et al., 2002; Trangadia et al., 2010; Chand and Chhabra, 2013). The disease has been reported from almost every state with significant seropositivity in animals. Various risk factors including lack of farm hygiene, overcrowding of animals and unrestricted movement of animals for trade purpose have been recognized in India (Chand and Chhabra, 2013; Saini et al., 1992).

The pace of change among dairy industry in India has resulted in its intensification and animal movements. This provides a new and increased potential risk in spreading the infection (Renukaradhya et al., 2002). The long term serological studies at national level indicated that about 5% cattle and 3% buffalos could be infected with brucellosis (Renukaradhya et al., 2002). Close proximity of farmers with animals in rural areas and consumption of unpasteurized dairy products lead to cases of brucellosis. Seroprevalence among occupationally exposed individuals and PUO cases has been demonstrated in almost all states of India. Prevalence of brucellosis in organized farm with abortion storms in Goa region was investigated by Barbuddhe et al. (2004). Out of 107 serum samples tested for brucellosis 40 (37.38%), 36.9 (36.45%) and 43(40.78%) were found to be positive for antibodies against *Brucella* by RBPT, STAT and AB-ELISA, respectively.

Presently, no effective vaccines are avaialble for humans against brucellosis. The disease can be eliminated only by eradicating it from animals. An improved epidemiological survey is needed to find out the magnitude and extent of brucellosis both in humans and animals. Detailed molecular characterization of species and biotypes is necessary for sustainable management of the disease. It is customary to

know how many clonal groups are prevalent in the region. Looking to the present scenario, it is utmost necessary to know the status of prevalence of *Brucella* to prevent the spread of infection. Improper hygiene maintenance in livestock farms may lead to *Brucella* infection which may spread to humans. Also, the state of Goa has many organized farms providing animal products and a large numbers of people live in close contact with dairy animals. Therefore, the study was proposed to determine the prevalence and diversity of *Brucella* among livestock and human populations of Goa. The study was proposed with the following objectives -

1. To determine the prevalence of *Brucella* among livestock and suspected humans by serological analysis
2. To isolate and characterize *Brucella* from serologically positive livestock and humans
3. To study biotypes and virulence profiles of *Brucella* isolates
4. To study the risk factors associated with brucellosis

Chapter 2
Review of Literature

2.1 Brucellosis

2.1.1 in humans

Brucellosis in humans is manifested usually as an acute or sub- acute febrile illness. It may mimic a large number of other infectious and non-infectious diseases presenting as an influenza-like or septicemic illness. The infection may persist and progress as chronically incapacitating disease. In humans brucellosis causes undulating fever, weight loss, sweating, chills, headaches, depression, low backache, muscle and joint pain (Dossey, 2010). The frequent focal complications of osteoarticular manifestations include spondylitis, sacroiliitis, or arthritis (Colmenero et al., 1996; Gür et al., 2003). Inadequate or delayed treatment may lead to severe and incapacitating persistent infection with enduring consequences, and may cause complications leading to endocarditis and neurobrucellosis.

The incubation period of the disease is two to three weeks with acute nature in about half the cases. Acute brucellosis accompanied by bacteremia helps to spread the pathogen to various organs (Greenfield et al., 2002). Survival and replication of *Brucella* spp. in mononuclear phagocytic cells may result in the high occurrence of protracted disease, and complications. Clinical signs in human brucellosis may vary with the system of involvement such as osteoarticular, dermal, gastrointestinal, cardiovascular, and neurologic symptoms. Pyrexia of unknown origin with a severe onset may also develop (Saltoglu et al., 2004). Patients usually show undulating fever (>38.5°C) (Young, 1995).

Hepatomegaly and splenomegaly may be manifested in about one-third of patients. Lymphadenopathy and osteoarticular manifestations account for major focal complications (Franco et al., 2007). Diverse neurological findings may be manifested

(Franco et al., 2007). Complication leading to endocarditis may account for 5% total mortality rate (Uddin et al., 1998).

Brucellosis in children needs special mention, as it can be overlooked in infants (Tsolia et al., 2002). In children monoarticular arthritis is common osteoarticular finding. Transmission of human brucellosis vertically (Giannacopoulos et al., 2002, Palanduz et al., 2000), via breast milk (Barroso et al., 1998; Celebi et al., 2007) and blood transfusion have been reported (Al-Kharfy, 2001; Doganay et al., 2003). Brucellosis may cause spontaneous abortions in humans; however, less frequently than do other bacterial infections (Young et al., 1983) may be because of lack of the sugar, erythritol in the human placenta and fetus (Poole et al., 1972). Though rare, cases of congenital brucellosis due to transplacental exposure have been reported (Glocwicz et al., 2010). Recently, sexual transmission of *B. melitensis* has been demonstrated in 2 cases (Meltzler et al., 2010).

2.1.2 In bovines and other animals

Brucella abortus has been usually reported to cause bovine brucellosis. However, rarely *B. melitensis* and *B. suis* may also cause brucellosis in bovines (OIE, 2009). The disease is documented by abortions typically in last trimester of pregnancy, retention of placenta, endometritis, still births and reduction in milk yield (Radostits et al., 2007). Usually, infected cows abort only once, in subsequent pregnancies, calving may be normal with heavily infected retention of placenta (Morgan, 1969).

Some infected cows do not show symptoms of the disease and calves born are normal (Nicoletti, 1980). The brucellae get localized in the mammary glands and associated lymph nodes of about 80% of the animals infected with brucellosis and excrete the organisms in milk throughout their lives (Hamdy and Amin, 2002).

The mechanisms of *Brucella*-induced abortions are poorly understood. Ruminant placentation is composed of multiple placentomes, which are formed by the combination of the maternal caruncular endometrium and fetal cotyledon. The presence of erythritol, a four-carbon polyol, in fetal tissues of ruminants has been demonstrated (Keppie et al., 1965). Growth of *B. melitensis* and *B. abortus* has been shown to be stimulated by high concentrations of erythritol (Keppie et al., 1965). Placentitis, which prevents the delivery of nutrients to the fetus and results in fetal stress and death, has been hypothesized as being responsible for abortion in brucellosis. However, experimental confirmation of this hypothesis has not been reported (Thoen et al., 1993).

Infected bulls may develop systemic signs of infection including fever, anorexia, and depression, although infection is often inapparent (Campero et al., 1990). The most significant lesion induced by *B. abortus* in bulls is orchitis (Lambert et al., 1963; Trichard et al., 1982) coupled with seminal vesiculitis and epididymitis (Rankin, 1965; McCaughey and Purcell, 1973). Affected bulls may develop permanent infertility due to chronic orchitis and fibrosis of the testicular parenchyma (Campero et al., 1990). Occasionally carpal hygromas abscess and arthritis can be observed in both sexes.

Brucellosis in caprines and ovines is mainly caused by *B. melitensis* and occasionaly by *B. abortus* (OIE, 2009). Arthritis is rarely observed in *B. melitensis* infected sheep and goats. Shedding of *B. melitensis* in camel milk has been reported. In horses, infection is often asymptomatic; however, local abscess formation in bursae may be observed (Corbel, 2006).

In pigs, infection by *B. suis* causes chronic inflammatory lesions in the reproductive organs (OIE, 2009). Of the five biovars of *B. suis*, the infection in pigs is

caused by biovars 1, 2 or 3 with varied geographical distribution and pathology (OIE, 2009). Biovars 1 and 3 are highly pathogenic for humans leading to severe disease. Skin abscesses, discospondylitis, hepatic and splenic necrosis, meningitis, and abortion have been observed in marine mammals suffering from brucellosis (Foster et al., 1996).

2.2 Pathogenesis and virulence factors

The ability of *Brucella* spp. to cause disease requires a few critical steps during infection. *Brucella* spp. can invade epithelial cells of the host, allowing infection through mucosal surfaces: M cells in the intestine have been identified as a portal of entry for *Brucella* (Ackermann et al., 1988; Paixão et al., 2009). The mechanisms that allow host cell invasion by *Brucella* spp. are not completely clear. Specific host receptors that interact with *Brucella* have not yet been identified, internalization of *Brucella* into host cells requires cytoskeletal changes (Guzmán-Verri et al., 2001 Pizarro-Cerdá et al., 1999). Interestingly, invasion through the digestive tract does not elicit any inflammatory response from the host (Paixão et al., 2009). Therefore, *Brucella* spp. attacks the innate immune system of the host silently.

Brucella spp. may prevent activation of the host innate immune system (Barquero-Calvo et al., 2007). Maturation of dendritic cells and production of pro-inflammatory cytokines is hindered by Btp1/TcpB. Ubiquitination and degradation of Mal (MyD88 adaptor-like) is mediated by Btp1/TcpB by entering the host cytosol by an unknown mechanism. Mal is an adaptor required for both TLR2 and TLR4. This leads to proteasomal degradation and inhibition of signaling via TLR2 and TLR4 (Atluri et al., 2011). Once *Brucella* spp. has invaded, usually through the digestive or respiratory tract, they are capable of surviving intracellularly within phagocytic or non-phagocytic host cells (Carvalho Neta et al., 2010). *Brucella* has the ability to

interfere with intracellular trafficking, preventing fusion of the *Brucella*-containing vacuole (BCV) with lysosome markers, and directing the vacuole towards a compartment that has rough endoplasmic reticulum (RER), which is highly permissive to intracellular replication of *Brucella* (Anderson et al., 1986; Pizarro-Cerdá et al., 1998; Pizarro-Cerdá et al., 2000).

Brucella spp. lack majority of the classical bacterial virulence factors including exotoxins, cytolysins, capsule, fimbriae, flagella, lysogenic phagesand host cell apoptosis inducers (Moreno and Moriyón, 2006). Lipopolysaccharide (LPS) of *Brucella* helps for initial survival of bacteria in macrophages (Lapaque et al., 2005). Functional and structural integrity of the outer membrane of Gram-negative bacteria is maintained by LPS. The surface-exposed O-polysaccharide which determines the smooth or rough LPS phenotype of *Brucella* spp., is important in virulence related with smooth LPS. Smooth LPS has role in the early development of the *Brucella*-containing phagosome.

Brucella can block maturation of phagosome, resulting in inhibition of phagosome-lysosome fusion through the interaction of smooth LPS with lipid rafts (Porte et al., 2003). Smooth LPS provides resistance to complement and antimicrobial peptides (defensins and lactoferrins) (Martínez de Tejada et al., 1995; Fernandez-Prada et al., 2001; Lapaque et al., 2005). The genes encoding the enzyme urease, involved in nitrogen metabolism and causing increase of pH due to ammonia production are necessary for infection by *B. suis, B. abortus* and *B.melitensis* (Paixão et al., 2009; Bandara et al., 2007; Sangari et al., 2007).

The BvrR/BvrS two-component regulatory system is another important virulence mechanism of *Brucella.* This is required for modulation of the host cell cytoskeleton upon *Brucella* invasion, and for regulation of the expression of outer

membrane proteins, some of which are required for full virulence (López-Goñi et al., 2002). Cyclic β-1,2-glucans of *Brucella* avoid fusion of the bacteria-containing vacuole with lysosomes in macrophages . Phagosome maturation is prevented by cyclic β-1,2-glucans by interfering with lipid rafts which alter protein expression in the vacuolar membrane and exclude lysosomal proteins (Starr et al., 2008; Arellano-Reynoso et al., 2005; Briones et al., 2001). *Brucella* spp. expresses a type IV secretion system (T4SS) which is crucial for intracellular survival in host cells (O'Callaghan et al., 1999; Hong et al., 2000). The *Brucella* T4SS secrets effector molecules which are necessary in phagosome maturation and transferring of the *Brucella*-containing vacuole towards its replication niche (Boschiroli et al., 2002).

Similarly, a virulence factor of *Brucella*, PrpA, a T-independent B cell mitogen, modulates the host immune response during *Brucella* infection (Spera et al., 2006; Spera et al., 2013). A mutant of *B. abortus* lacking Omp25 which was attenuated in pregnant heifers failed to replicate in bovine phagocytes and trophoblasts suggesting the role of other outer membrane components as virulence factors of *Brucella* spp. (Edmonds et al., 2001). The role of the *bvfA*, *a* small periplasmic protein has been suggested to be essential for the virulence of *Brucella suis* (Lavigne et al., 2005).

2.3 Diagnosis of brucellosis

2.3.1 Serology

Brucellosis was first diagnosed in 1897 by a serological test developed by Wright and Smith (Wright and Smith, 1897) involving a simple tube agglutination test. Serological tests are regularly used in prevention, control, eradication, and surveillance programmes. There are substantial discrepancies in the accuracies of the different serological tests varying considerably and use of a panel of tests has been

recommended for diagnosis (Poester et al., 2010). Traditionally, though screening tests are inexpensive, fast and sensitive; however, lack specificity. Confirmatory tests need to be both sensitive and specific. False positive reactions can be eliminated to some extent by use of confirmatory tests. Practically, confirmatory tests are complex and costlier to execute as compared to the screening tests (Diaz et al., 2011).

Common epitopes in the OPS within the LPS among the smooth *Brucella* species are shared, therefore, all serological tests use *B. abortus* antigen in the form of whole cells, SLPS or OPS (OIE, 2009). The OPS component is absent in *B. ovis* and *B. canis,* therefore, R-LPS (rough LPS) is generally employed as an antigen for detecting the antibodies against *B. ovis* and *B. canis* (Blasco, 1990; Carmichael, 1990). Most recently developed tests use either SLPS or OPS antigens although some attempts at using protein antigen have been made.

Cattle infected with *B. abortus* generally produce an early IgM isotype antibody response, the amplitude of which is governed by a multiplicity of factors. It usually appears 5 to 15 days post exposure but may be delayed (Beh, 1973; Beh, 1974; Allan et al., 1976). The IgM antibody responses are succeeded shortly by IgG1 isotype of antibody response and consequently by IgG2 and IgA (Corbel, 1972; Levieux, 1978; Nielsen et al., 1984). The IgM response would be more suitable to measure as an indicator of exposure as this isotype initiates early. However, a variety of other microorganisms has epitopes on antigens similar as OPS and produce cross-reacting IgM antibody (Corbel et al., 1985).

Therefore, measurement of IgM antibody may result in a false positive reaction in serological tests. False positive reactivity would lead to specificity problems which would be of considerable consequence in an early control programme resulting in unnecessary slaughter; in the last stages of an eradication programme and

in free areas, resulting in expensive follow-ups. Owing to the late occurrence of IgG2 and IgA isotypes in infection, their assay lowers the sensitivity of the test. Therefore, tests that detect IgG1 isotypes are useful for serological testing for brucellosis (Allan et al., 1976; Nielsen et al., 1984; Lamb et al., 1979; Butler et al., 1986).

Tentative diagnosis of brucellosis can be done by revealing specific antibodies in serum or milk samples. However, a number of bacteria, namely, *Yersinia enterocolitica* O: 9, *Francisella tularensis*, *Vibrio cholerae*, *Escherichia coli* O: 157, *Salmonella urbana* group N, and *Stenotrophomonas maltophilia* cross react with *Brucella* (Al Dahouk et al., 2003). Many of the serological tests are less sensitive as the tests have ability to interact secondarily as to agglutinate antigens or complement fixation.

A battery of serological tests have been developed for diagnosis of brucellosis such as milk ring test, STAT, RBPT, buffered plate agglutination test and Enzyme linked immunosorbent assay. The RBPT has been used as a quick screening test for surveillance of the disease in field cases amongcattle in areas endemicfor the disease. Rose Bengal dye stained 8% suspension of *B. abortus* strain 99 or strain 1119-3 (USDA) buffered to pH 3.65±0.05 is used as the antigen for RBPT. Acidification of the antigen helps to loosen the bond between the antigen and non-specific agglutinins resulting in improved specificity and sensitivity of the RBPT and the BPAT (pH 3.7±0.03) as compared to SAT (Alton et al., 1975). The acidic pH diminishes agglutination by IgM, however, it promotes agglutination by IgG1, reducing cross-reactions (Allan et al., 1976; Corbel, 1972)

SAT (Serum Agglutination Test) is also known as STAT (Standard tube agglutination test). STAT is performed by mixing whole bacterial cell antigens with the serum and incubating the mixture. The test detects primarily the IgM isotype as it

is done at a near neutral pH. The test is sensitive, however, as it detects IgG less efficiently, especially IgG1, resulting in low assay specificity (Corbel et al., 1985; Nielsen et al., 1984; Rice and Boyes 1971). Therefore, the SAT is generally used in combination with other tests rather a single test. In human beings, SAT titre in excess 1:160 coupled with clinical presentation is considered to be presumptive case. In endemic areas, titers more than 1:320 are regarded as more specific (Smits and Kadri, 2005).

An adaptation of the SAT, which involves the addition of EDTA, has proved to significantly increase the test specificity by reducing the chances of cross-reactions. It can, therefore, be used as an alternative to routine SAT (Poiester et al., 2010; Kaltungo et al., 2013). Dithiotreitol and 2-mercaptoethanol which reduce the disulphide bridge of IgM allowing detection of mainly IgG have both been added in SAT for the serological diagnosis of brucellosis. However, IgG can also be reduced in the process, giving false negative results. Though in general, reduction of IgM increases specificity (Poiester et al., 2010).

Precipitation of high molecular weight serum glycoproteins removes non specific reactivity in agglutination. In rivanol precipitation test, rivanol (2-ethoxy-6, 9-diaminoacridine lactate) is added to serum, the precipitate is removed by centrifugation. Serum (diluted or undiluted) can be further tested by aquick plate agglutination test or a tube test. As the precipitation tests are laborious, therefore, usually used as confirmatory tests (Nielsen, 2002; Poiester et al., 2010). The test is capable of distinguish between vaccinated and infected animals, and also chronic carriers. Interpretation of the test is, however, difficult.

The milk ring test (MRT) is performed by mixing haematoxylin stained *Brucella* cells with whole milk or whole milk with cream (Huber et al. 1986; Hunter

and Allen, 1972; Sutra et al., 1986).Anti-*Brucella* antibodies present in the milk get attached to fat globules and the Fc portion of the immunoglobulins attach to antigen resulting in appearance of a purple band in the cream layer. In case of absence of antibodies in milk, the fat layer remains a buff colour with evenly distributed purple antigen throughly in the milk. The test can be used to test the animals individually as well the pooled milk samples. False reactions in the milk ring test may arise by abnormal milk such as mastitic milk, colostrum and late lactation cycle milk. The milk ring test can be used as inexpensive preliminary test in combination with others.

For diagnosis of chronic cases of brucellosis or in vaccinated animals where SAT titres are inconclusive, CFT has been preferred (Nicoletti and Muraschi, 1966). Being the most sensitive and specific conventional serological method, CFT is recommended as confirmatory test for diagnosis of brucellosis (Alton et al. 1975; Nielsen et al., 1995).

Various antigens namely, whole cells, sLPS, native hapten polysaccharide, autoclaved antigen, OMPs and cytoplasmic proteins have been tried for indirect enzyme linked immunosorbent assay (iELISA) (Al Dahouk et al., 2003). However, though sensitive, ELISAs are prone to false positive reactions as the tests use bacterial extracts containing LPS in large high quanties. Antibodies to cross-reacting bacteria or vaccination may result in false-positive reactions. Competitive enzyme immunoassays (cELISAs) can differentiate between antibodies due to vaccination and natural infection and are less cross-reacting. (Nielsen et al., 1995; Nielsen et al., 1996) and can be employed as confirmatory or screening test due to their higher sensitivity. For diagnosis of chronic and convalescent cases, the SAT is less sensitive than ELISA, whereas, both the tests demonstrate similar results in acute cases. The detection of the IgG isotypes by ELISA is more sensitive than the detection IgM

isotype. The fluorescence polarization assay (FPA) is based on depolarization of light more rigorously by a rapid rotating small molecule than by a bigger molecule (Nielsen et al., 1996). Every molecule in a solution is in state of random rotation. The rate of rotation is inversely proportional to the size of the molecule. The rate of rotation of a small antigenic molecule may change after attachment with antibodies which is measured using a fluorescent label and polarized light. The FPA can easily discriminate between vaccinated animals from naturally infected animals as compared to cELISA (Nielsen et al., 1996) and its specificity in vaccinated animal increases with the period passed after injection (Samartino et al., 1999). The FPA is rapid taking a few minutes to perform with minimal preparation and even using whole blood samples in field conditions (Nielsen and Gall, 2001).

The lateral flow assay which is easy-to-perform is suitable for rapid field or point of care testing in resource poor endemic areas in absence of well equipped laboratories. The test has been confirmed to be more sensitive than the SAT in complicated and chronic cases (Zeytinoğlu et al., 2006).

2.3.2 Isolation of *Brucella*

2.3.2.1 Culture media

Though fastidious, *Brucella* can grow on most of the standard laboratory media including blood agar, chocolate agar, trypticase soy agar (TSA) and serum-dextrose agar (SDA). A variety of dehydrated basal media are available commercially such as *Brucella* basal medium, tryptose (or trypticase) soy agar (TSA). Bovine or equine serum (2-5%) is added to basal media to support the growth of various strains. The agar plates inoculated with the samples need to be incubated aerobically at 35-37°C in presence of 5-10% CO_2. In case of isolation of *Brucella* from highly contaminated samples like faeces or infected tissues, basal media are required to be

supplemeted with antibacterials such as bacitracin, amphotericin B, cycloheximide/natamycin, d-cycloserine, nalidixic acid, polymyxin B and vancomycin (Farrell, 1974). The widely used selective media for *Brucella* are the Kuzdas and Morse (Kuzdas and Morse, 1953) and the Farrell´s medium (Farrell, 1974). Use of Modified Thayer-Martin medium has been suggested together with Farrell's as later has been found to inhibit some strains of *B. abortus*, *B. melitensis*, and *B. ovis*.

It has been reported that, usually, the clinical samples like milk, clinical samples and dairy products contain low number of *Brucella* organisms. In these cases, the samples need to be enriched using liquid media. The broth media used for enrichment includes serum–dextrose broth, tryptose (or trypticase)-soy broth and *Brucella* broth supplemented with antibiotics. A nonselective, biphasic, Castaneda's medium is widely employed for borth culture of *Brucella* (Castaneda, 1947).

2.3.2.2 Isolation from animals

The presence of brucellosis in infected animals may be confirmed by culturing the pathogen from clinical samples collected from infected animals (aborted fetuses, vaginal secretions, blood and milk). Brucellae can also be isolated from vaginal swabs, discharges from uterus, stomach contents, spleen, and lung collected from aborted foetuses, placental bits, semen, and synovial fluids (OIE, 2009). Associated lymph nodes and spleen, udder tissues, testes, epididymes are useful samples for culture from animal carcasses (Liu, 2009).

2.3.2.3 Isolation from humans

Blood culture is commonly performed in patients with undulant fever. The organism may also be isolated from pus, tissue samples, and cerebrospinal, joint, or ascitic fluids (Etemadi et al., 1984; Doern, 2000). As *Brucella* is frequently present in

low numbers, broth or a biphasic medium (Castañeda) is favored to culture blood and other body fluids. Blood from infected patients collected in heparin or 1% sodium citrate need to be inoculated in liquid medium. Later, the medium is streaked on solid medium and examined for colonies. The sluggish growth of *Brucella* in primary cultures may delay the diagnosis for more than a week (Yagupsky, 1999). Also, sensitivity of blood-culture is usually low (50 to 90%). It depends upon the stage of disease, the species, the medium used, bacterial load and the method used (Mantur and Mangalgi, 2004; Yagupsky, 1999).

The conventional method for culture of *Brucella* spp. from clinical samples is the biphasic Ruiz-Castañeda technique (Yagupsky, 1999; Ganado and Bannister, 1960; Ruiz-Castañeda, 1947), however, the techniques has been replaced by automated culture systems (Corbel, 2006; Cockerill et. al., 1997; Mantur and Mangalgi, 2004). The newer techniques have increased sensitivity and reduced times for culture (Durmaz et. al., 2003; Bannatyne et. al., 1997; Navas et. al., 1993). The time taken for detection has been curtailed significantly with the advent of the newer semiautomatic methods (BACTEC 9204 and BacT/Alert). Contaminated aerosols may infect laboratory personnel particularly when using the lysis centrifugation method, therefore, special care must be taken to avoid infection (Yagupsky and Baron, 2005).

2.3.3 Identification

2.3.3.1 Direct microscopic examination of stained smears

Brucella spp. can be demonstrated in stained smears by direct microscopic examination. The smears can be prepared from fetal and placental tissues, vaginal swabs and semen. The commonly used methods include the Stamp's modification of the Ziehl-Neelsen's method and the modified Köster (Alton et al., 1975). Brucellae are

coccobacillary or short rods measuring 0.6-1.5 μm long and 0.5-0.7 μm wide. Though not truly acid-fast, brucellae stain red against a blue background and are not decolourized using weak acids. *Coxiella burnetii* and *Clamydophila abortus* may apparently look like *Brucella* (Alton et al., 1975).

2.3.3.2 Biochemical tests

Brucella organisms can be identified based on colony morphology, Gram staining, agglutination with anti-*Brucella* serum, urease, catalase and oxidase production. Brucellae are Gram-negative, appear faintly stained and resemble "fine sand" microscopically. The smooth strains of *Brucella* are transparent and pale yellow on growth media. Smooth strains look like droplets of honey with a shiny surface when seen in transmitted light. Rough colonies are more opaque and have a granular surface (Braun and Bonestell, 1947). A yellow uniform suspension is produced by smooth colonies, whereas, granular agglutinates are shown by rough colonies. The variation in colonies can be seen by observing the plates with crystal violet stained colonies under oblique light (White and Wilson 1951).

2.3.3.3 Identification by PCR

Standard PCR and Multiplex PCR: The identification of *Brucella* at genus, species and even biovar levels has improved with the application of the PCR. A number of PCR methods developed for the detection of *Brucella* are more and more used in the diagnosis of brucellosis owing to their more sensitivity than conventional culture methods and more specificity than serological methods (Al Dahouk, 2013). Also working with DNA reduces risk of acquiring infection. Initially, PCR methods have been developed for identification of bacterial pathogens; however, these assays can be used for detection of the organisms in human and animal clinical samples (Smirnova et al., 2013).

PCR had been successfully used for the identification of *Brucella* in bovine blood and milk (Leal-Klevezas et al.1995; Romero and López-Goni, 1999; Romero et al., 1995), organs of naturally infected cattle (Gallien et al., 1998; O'Leary et al., 2006) and goats or sheep milk and cheese (Serpe et al., 1999; Tantillo et al., 2001; Tantillo et al., 2003). For identification of *Brucella* spp. at the genus- level, the primers for sequences encoding *bcsp31* (B4/B5) (Baily et al., 1992), 16SrRNA (F4/R2) (Romero et al., 1995), 16S-23S intergenic transcribed spacers (ITS) (Rijpens et al., 1996; Bricker, 2000), 16S-23S rDNA interspace region (ITS66/ITS279) (Keid et al., 2007), *IS711* (IS313/IS639) (Hénault et al., 2000), *per* (bruc1/bruc5) (Bogdanovich et al., 2004), *omp2* (JPF/ JPR) (Leal-Klevezas et al., 1995), outer membrane proteins (Imaoka et al., 2007), proteins of the omp25/omp31 family (Vizcaino et al., 2004) have been used.

The 16S-23S genes, the *IS*711 insertion sequence and the *bcsp*31 gene of *Brucella* spp. are validated for detection of *Brucella* (Ouahrani-Bettache et al., 1996). Comparison of sensitivity of 3 pairs of primers amplifying 3 different fragments including a gene encoding the *bcsp31*, a sequence of 16S rRNA of *B. abortus*, and a gene encoding *omp2* revealed the sensitivities of the *bcsp31*, omp2 and 16S rRNA to be 98%, 88.4% and 53.1%, respectively (Baddour and Alkhalifa, 2008). Navarro et al. (2002) compared detection ability of three primer pairs specific for the *bcsp31*, 16SrRNA and *omp2* genes of *Brucella* in human blood samples and variation in sensitivity for detecting purified *Brucella* DNA was reported with the *bcsp31* gene to be the most sensitive for detecting *Brucella* DNA.

The AMOS-PCR (*abortus-melitensis-ovis-suisi*) was the first species-specific multiplex PCR used to identify and differentiate *Brucella* biovars. It is based on polymorphism due to species-specific localization of the insertion sequence *IS711* in

the *Brucella* chromosome (Bricker and Halling, 1994). The assay was improved to identify the vaccine strains S19 and RB51 by including further strain specific oligonucleotides into the reaction mixture (Bricker and Halling, 1995).

The AMOS PCR assay was further modified as BaSS-PCR (*Brucella abortus* Strain Specific PCR assay) which helped to identify and differentiate field strains of biotypes 1, 2 and 4 of *B. abortus,* and vaccine strains and other *Brucella* species from cattle (Bricker et al., 2003). For identification of *B. abortus* biovars 3, 5, 6 and 9 a new primer was developed (Ocampo-Sosa et al., 2005).

For recognition and discrimination of *Brucella* species and vaccine strains in a single step, a multiplex PCR assay, Bruce-ladder was developed (García-Yoldi et al., 2006). The PCR was further enhanced to identify the marine strains, *B. microti* and *B. inopinata*. However, it does not differentiate at the biovar level, or below (López-Goñi-Goni et al., 2008; Hubber et al., 2009; Mayer-Scholl et al., 2010). A multiplex PCR assay (Suis ladder) was developed recently to differentiate among biovars of *B. suis* (López-Goñi et al., 2011). A comparable multiplex approach was described having ability to discriminate the six classical species based on species-specific differences (Hinic et al., 2008), however, the approach was not as inclusive as 'Bruce-ladder'.

Real time PCR: - Nucleic acids can be quantified in individual samples and data can be automated using real time PCR. It can be performed in a short time without electrophoretic analysis, and avoiding contamination. Recently, real-time PCR assays targeting 16S-23S internal transcribed spacer region (ITS) and the genes coding o*mp25* and *omp31* (Kattar et al., 2007), *bcsp 31* (Colmenero et al., 2005; Debeaumont et al., 2005; Queipo-Ortuño et al., 2008), and *IS711* (Cerekci et al., 2011; Zhang et al.,

2013) have been developed for the rapid detection and differentiation of *Brucella* species in clinical samples.

2.4 Epidemiology of brucellosis

2.4.1 Global Distribution

Brucella species and biovars show variation in their geographic distribution (Hirsh, 2003). *Brucella abortus* has been reported worldwide in cattle-raising regions. The pathogen has been eradicated from Japan, Canada, and some European countries, Australia, New Zealand and Israel (OIE, 2009) and nearly eradicated from domesticated herds in the U.S.A., still the persistence of *B. abortus* has been reported in wildlife hosts in the Greater Yellowstone Area of North America. . Distribution of *B. abortus* biovar 1 and *B. abortus* biovar 2 has been reported worldwide, on the other hand, *B. abortus* biovar 3 has been observed predominantly in Italy, India, Egypt, and Africa and *B. abortus* biovar 5 in Germany and United Kingdom (Hirsh, 2003), but has also been observed in France (Garin-Bastuji, 1993). *B. abortus* biovars 4 and 6 have been documented less frequently than biovars 1, 2, and 3 in Mexico and France (Garin-Bastuji, 1993; Luna-Martínez, 2002).

Detection of *B. melitensis* has never been reported from some countries. Its eradication from small ruminants has also not been reported reliably in any country (Robinson, 2003). The occurrence of *B. melitensis* is widespread in the Mediterranean, the Middle East and Central Asia, around the Persian Gulf and in some countries of Central America. The pathogen has also been reported from Africaan countries and India. It is not endemic in northern Europe, North America (except Mexico), Southeast Asia, Australia or New Zealand.

The occurrence of *B. ovis* has been reported mostly from sheep-rearing regions of the world particularly, Australia, New Zealand, North and South America, South

Africa and many European countries (Spickler, 2009). The U.S., Canada, and many European countries have achieved eradication of *B. suis* from domesticated pigs. However, *B.* suis has been prevalent in wild or feral swine herds in the U.S., Europe and Queensland, Australia. Infrequent outbreaks of *B. suis* infection from wild life sources have been reported in domesticated herds and humans (Spickler, 2009).

2.4.2 Outbreaks of brucellosis

2.4.2.1 In Animals

B. abortus biovar 2 outbreak was reported in cattle in Alberta (Forbes et al. 1989). An outbreak of bovine brucellosis was reported in County Clare, Ireland, during 2005 (Hayes et al., 2010). A brucellosis outbreak was reported in an organized dairy farm which had experienced abortions, retaintion of placenta and stillbirths among 24 of the 290 animals in the farm in Himachal Pradesh (Chahota et al., 2003). Outbreak of bovine brucellosis in about 20 buffaloes in Uttar Pradesh was described (Jain et al., 2013).

2.4.2.1 In Humans

Outbreak of brucellosis in humans was reported in Chouf district of Lebanon in 2009 (Al Shaar et al., 2014). Rodrigues et al. (2013) reported outbreak of laboratory-acquired infection due to *B. abortus* in Brazil. Three cases were confirmed serologically. Román et al. (2013) reported cases of food-borne brucellosis at a Peruvian police officer cafeteria. Active surveillance estimated the attack rate at 26.5% (13 of 49). Blood cultures from four cases were positive for *Brucella*.

Human brucellosis outbreak due to consumption of locally produced raw cheese was reported by Karagiannis et al. (2012) in Thassos, Greece. Seven cases of human brucellosis were detected and *B. melitensis* biotype 3 was identified in two clinical samples. Outbreak of brucellosis acquired through camel milk consumption

was observed in humans in southern Israel (Shimol et al., 2012). Gyuranecz et al. (2011) described the first known outbreak of *B. canis* infection introduced to the kennel consisted of 31 dogs of various ages likely by a pregnant female in Hungary in 2009 (Gyuranecz et al., 2011).

Marković-Denić et al. (2010) described the first outbreak of brucellosis in humans and animals in the region of Sabac, Serbia. Twelve cases of brucellosis were recorded. Castell Monsalve et al. (2009) described 3 outbreaks of brucellosis during 1-year period in Spain. Two of these outbreaks, with 2 cases each occurred in 2 cheese factories and the third outbreak, also with 2 cases, occurred in a laboratory belonging to the local government. An uncommon outbreak of brucellosis caused by *B. canis* involving six persons, a bitch and three puppies was observed by Lucero et al. (2010). Brucellosis outbreaks originated from elk in the Greater Yellowstone area were investigated by Beja-Pereira et al. (2009). Farina et al. (2008) described an outbreak of *Brucella* infection attributed to cheese from an area of Italy was reported in Treviso province during August 2005. Nine cases of brucellosis were identified in an outbreak from unpasteurized raw milk in Moroccan immigrants in Spain (Ramos et al., 2008). A sporadic outbreak of human brucellosis involving 11 cases has been reported by Park et al. (2005) in Korea. Méndez Martínez et al. (2003) reported an outbreak of brucellosis due to consumption of unpasteurized raw goat cheese in Andalucia, Spain. Kalla et al. (2001) investigated an outbreak of polyarthritis with 15% seropositive patients in a village in Churu district of Rajasthan.

Outbreak of brucellosis among farm workers with high rates of *B. melitensis* infection linked to an epidemic of caprine abortions in Argentina was reported (Wallach et al., 1997). Similarly, an outbreak of brucellosis among

employees involving 18 cases at a local pork processing plant at North Carolina were reported (CDC, 1994).

Four cases of laboratory-acquired brucellosis among technicians (Martin-Mazuelos et al., 1994) and eight cases among employees working at a microbiology laboratory were reported (Staszkiewicz et al., 1991). Wallach et al. (1994) observed an outbreak of brucellosis caused by *B. melitensis* in a family who consumed unpasteurized goat cheese. An outbreak of *B. melitensis* infection involving seven cases was linked to Italian pecorino cheese (Galbraith et al., 1969).

An outbreak of brucellosis attributed to *B. melitensis* was reported among persons of a predominantly Hispanic locality in Houston (Thapar and Young, 1986). The outbreak was traced to unpasteurized goats' milk cheese Nelson et al. (1975) recorded an unusual outbreak of laboratory acquired brucellosis in 3 persons due to contact with spilled *Brucella* culture.

2.4.3 Seroprevalence among bovines

Studies across the world demonstrate variable prevalence rates. Poulsen et al. (2014) observed overall apparent prevalence of 5.5% among cattle of northern Ecuador by RBPT. Screening of cattle sera by indirect ELISA and their confirmation by competitive ELISA revealed herd and individual seroprevalences to be 4.1 and 2.0 %, respectively in Tajikistan (Lindahl et al., 2014). Zolzaya et al. (2014) reported that overall apparent seroprevalence of brucellosis was 16.0% in cattle among two provinces in Mongolia. Sylla et al. (2014) investigated the prevalence of bovine brucellosis in Guinea by RBPT and CFT and was found to be 12% and 5.33 %, in Macenta and Yomou, respectively. Similarly, overall prevalence of 2.77% was observed in dairy cattle of Eritrea by RBPT and CFT (Scacchia et al., 2013). Maurice et al. (2013) observed overall seroprevalence of 9.6% in northern plateau of north

central Nigeria. Boukary et al. (2013) observed 1.3% animal level prevalence by indirect ELISA in Niger. Sero-epidemiological survey of brucellosis in cattle employing RBPT and CFT revealed apparent seroprevalences of 1% at the animal level and 4.9% at the herd level in western Ethiopia (Adugna et al., 2013).

Tschopp et al. (2013) observed overall prevalence of 1.7% among cattle in Ethiopia by ELISA. Islam et al. (2013) reported overall sero–prevalence of brucellosis as 21.36%.by RBPT and iELISA in Bangladesh. Tanner et al. (2014) reported seroprevalence of brucellosis as 17.72% and 27.42% among buffaloes at the Kruger National Park, South Africa using RBT and ELISA, respectively. Muflihanah et al. (2013) observed seroprevalence of 19.3% by CFT among beef cattle of Bali, Indonesia. *Brucella* antibodies were observed in 21.8% cattle herds in Peninsular Malaysia between 2000 and 2008 (Anka et al., 2013).

Overall prevalence of 2.4% and 4.7% by RBPT, and 1.2% and 3.34 % by indirect ELISA for *Brucella* antibodies was observed in the Nakasongola and Luwero districts (Nizeyimana et al., 2013). Chikweto et al. (2013) observed 6% cattle to be seropositive to *B. abortus* and/or *melitensis* by cELISA in Grenada, West Indies. Overall 8.6% and 5.7% of the animals were seropositive for brucellosis using RBT and cELISA, respectively in Nigeria (Cadmus et al., 2013). Brucellosis seroprevalence was found to be 20.7% by RBT and cELISA among cattle in Southern Province of Zambia (Muma et al., 2013). In Ethiopia, out of a total of 2334 cattle from 273 farms, 1.9% animals were seropositive using RBPT and CFT (Asmare et al., 2013). Herd-level and individual animal-level prevalences of brucellosis were reported to be 11.4% and 2.5%, respectively in Maranhão state, Brazil (Borba et al., 2013).

Kashiwazaki et al. (2012) reported 21.5% prevalence in 3 districts of west Uganda and 3.4% in 2 districts of east Uganda by RBPT and indirect ELISA. Overall seroprevalence of brucellosis in cattle was reported as 3·2% in dairy farms of Ethiopia by Asmare et al. (2013). In northern Nigeria, the overall animal-level prevalences in cattle were observed to be 37% by RBPT and 26.3% by using c-ELISA (Mai et al., 2012). Sanogo et al. (2012) reported that 10.3% of the cattle had antibodies against *Brucella* detected by RBT and indirect ELISA in savannah-forest region of Ivory Coast. Swai and Schoonman (2012) reported that overall seroprevalence of brucellosis among animals in Tanga city abattoir, Tanzania was 12%. Overall seroprevalence of brucellosis was reported to be 9.9% by RBT and C-ELISA in wildlife-livestock interface at the southeast lowland of Zimbabwe (Gomo et al., 2012).Antibodies against *B. abortus* were detected in 56.3% cattle by serum agglutination test in Punjab Province, Pakistan by Shabbir et al. (2011). Antibodies against *Brucella* were present in 3.5% cattle using RBPT as screening and CFT as confirmatory test in Ethiopia (Megersa et al., 2011). Makita et al. (2011) observed 5.0% prevalence of brucellosis among cattle in Kampala region, Uganda by cELISA. A study carried out at Addis Ababa dairy farms by Tesfaye et al. (2011) reported seroprevalenc of 2.5% and 1.5% by RBPT and CFT, respectively. The overall individual animal brucellosis seroprevalence in dairy cattle of Zimbabwe was reported to be 5.6% by RBPT and cELISA (Matope et al., 2011). Rahman et al. (2011) reported that 2.87% buffaloes, 2.66% cattle were positive by RBPT and indirect ELISA. Mohammed et al. (2011) observed that 4.04% and 3.86% cattle sera were positive by RBPT and cELISA respectively in Jigawa state, Nigeria.

In the smallholder dairy and traditional managed cattle, an overall seroprevalence of *Brucella* antibodies was reported to be 4.1% and 7.3%,

respectively by RBPT in North-eastern Tanzania (Swai and Schoonman, 2010). Seroprevalence rates of 6.00%, 6.17% and 5.31% were reported in the years 2004, 2005 and 2006, respectively using RBPT in Nigeria (Cadmus et al., 2010). The overall individual animal-level prevalence was reported as 4.9% by RBPT and CFT in cattle rearing areas of North West Ethiopia (Mekonnen et al., 2010). In Libya, Ahmed et al. (2010) observed 42% of cattle to be seropositive.

Ibrahim et al. (2010) reported an overall individual animal level prevalence of 3.1% using RBPT and CFT in Jimma zone, Ethiopia. The true prevalence of 6.5 % in individual cows was observed using RBPT and iELISA in Jordan (Al-Majali et al., 2009a). Seropositivity among 8.4% Holstein cattle was detected using ELISA in Cameroon (Bayemi et al., 2009). The occurrence of brucellosis was found to be 1.45 % in communal grazing areas of Kwa-Zulu, Natal (Hesterberg et al., 2008). In Turkey 35.30% and 32.92% and 39.45% cattle sera were found to be positive using RBPT, SAT and ELISA, respectively (Sahin et al., 2008). Seroprevalence of brucellosis was estimated at 23.9% by RBPT and cELISA in Zambia (Muma et al., 2007).

Rural farms (5.0%) were reported to have higher prevalence of *Brucella* antibodies than organized farm (2.5%) in Bangladesh (Amin et al., 2005). The mean seroprevalence of antibodies to *Brucella* was 3.3% using RBPT in Central African Republic (Nakouné et al., 2004). *Brucella* prevalence was found to be 7% by indirect ELISA in Chad (Schelling et al., 2003) and 4.7% in cattle and 4.2% in buffaloes by indirect ELISA in Srilanka (Silva et al., 2000). Prevalence of *B. abortus* by slow agglutination test (SAT) was found to be 14.1% in Dar es Salaam, Tanzania (Weinhäupl et al., 2000). Mean seroprevalence of brucellosis was found to be 6.6% among cattle in Akwapim-South district of Ghana by RBPT (Kubuafor et al., 2000).

2.4.4 Seroprevalence among bovines in India

Bovine brucellosis is common in most of the states in India. Long term serological survey across 23 states confirmed presence of antibodies in 19 states with variable prevalence rates (Renukaradhya et al., 2002). Many reports from other states are available supporting this study. Recently, Patel et al. (2014) reported overall herd and animal prevalence of 33.70% and 11.90%, respectively in peri-urban areas of Gujrat. Khajuria et al. (2014) observed that seroprevalence of brucellosis was 1.88% and 3.13% by RBPT and STAT, respectively among organized and unorganized farms in North India. Reddy et al. (2014) observed overall prevalence of 6.17% among slaughter cattle in Kerala using RBPT and cELISA. Chand and Chhabra (2013) observed 22.34% and 34.15% individual animal seroprevalence by RBPT and Indirect ELISA in Haryana and Punjab, respectively. Jagapur et al. (2013) found that 31.74% animals were positive for brucellosis, which included 27.21% cattle and 36.34% buffaloes among the 3 states of India. Sukumar et al. (2012) observed prevalence of 23.52% by RBPT; out of these 31.25% samples were positive by STAT in Tamilnadu. Londhe et al. (2011) reported 40.4% prevalence of brucellosis ranging between 26.31% and 70% in bovines in different districts of Maharashtra.

While testing a total of 30,437 bovine samples (23,284 cattle and 7,153 buffaloes), the prevalence rates of anti-*Brucella* antibodies were reported as 1.9% in cattle and 1.8% in buffaloes in a nationwide serological survey of brucellosis involving 23 states of India (Isloor et al., 1998). Trangadia et al. (2010) observed 22.18% and 13.78% seropositivity among bovines by ELISA and RBPT, respectively. Brahmabhatt et al. (2009) observed seroprevalence of 12.75% 11.16% and 19.12% by RBPT, STAT and i-ELISA, respectively in Gujrat. Upadhyay et al. (2007) observed that 7.25% bovine samples were positive by AB-ELISA, 4.73% by STAT and 2.90% by RBPT in Uttar Pradesh. Dhand et al. (2005) tested bovine

sera by AB-ELISA and reported 12.09% seroprevalence in villages of Punjab. While studying epidemiology of brucellosis in bovines in Punjab, the prevalences of 20.67% and 16.41% were observed in cattle and buffaloes, respectively by milk ELISA with an overall apparent prevalence of 18.26 % (Aulakh et al., 2008). Barbuddhe et al. (2004) reported seropositivity of 37.38%, 36.45% and 40.78% in cattle for antibodies against *Brucella* by RBPT, STAT and AB-ELISA, respectively in Goa.

Seropositivity for brucellosis was reported in 4.1% serum samples collected from cattle from 47 organised farms in Karnataka (Isloor et al., 1998). Within organised farms, with a history of reproductive disorders, the prevalence rate was 17%. In another study, serological - survey of bovine brucellosis in farms around Nagpur region indicated 9.7% seropositivity (Nawathe and Bhagwat, 1984). Seroprevalence of brucellosis was detected in 6.37% cattle and 4.9% buffaloes in the states of Uttar Pradesh and Delhi (Sharma et al., 1979).

2.4.5 Seroprevalence in humans

A study in Huye, Rwanda reported 25% women presenting with abortion/stillbirth positive for brucellosis by RBPT (Rujeni and Mbanzamihigo, 2014). Seroprevalence (6.4%) of brucellosis was reported among hunters and their families, butchers, health care workers, and those referred to medical diagnostic laboratories in Iran (Esmaeili et al., 2014). In the northwestern part of Ecuador, overall seroprevalence of brucellosis in humans was estimated as 1.88% (Ron-Román et al., 2014). Zolzaya et al. (2014) reported that overall apparent seroprevalence of brucellosis was 27.3% among humans in Mongolia. Brucellosis seorprevalence in people of Bangladesh was ranged between 2.5-18.6% based on occupation of people (Islam et al., 2013).

Aworh et al. (2013) observed a seroprevalence of 24.1% in among abattoir workers of Abuja, Nigeria. Of these, 59.3% were butchers, and 20.4% were meat-sellers. In Potohar plateau of northeastern Pakistan, Ali et al.(2013) reported overall seroprevalence of brucellosis as 6.9% among different occupational groups using RBPT and SAT. Similarly, seroprevalence of 14.4% was detected among individuals in at-risk professions in Wyoming (Luce et al., 2012). In an urban slum population of Brazil, seroprevalences for *B. abortus* and *B. canis* were reported to be 13% and 4.6%, respectively (Angel et al., 2012).

Seroprevalence among women with miscarriage was 1.8% and that with no miscarriage was 1% using RBPT and CFT in Jordan (Abo-shehada and Abu-Halaweh, 2011). In Kyrgyzstan, overall apparent seroprevalences of brucellosis was 8.8% among humans (Bonfoh et al., 2012). *B. canis* seropositivity rate in the healthy blood donors was 1.6% by 2-ME test in Turkey (Sayan et al., 2011). Seropositivity for brucellosis was detected in 5.4% suspected patients by ELISA in Malaysia (Jama'ayah et al., 2011). Prevalence of brucellosis was found to be 21.7% among high risk occupational group of Pakistan using ELISA (Mukhtar, 2010).

Seroprevalence of brucellosis was found to be 9% among abattoir workers by RBPT, modified RBPT (mRBPT), SAT and cELISA in Sudan (Omer et al., 2010). In Iran, significantly higher positive brucellosis serology was observed in HIV-infected patients than in controls (73.3% vs. 30%) (Abdollahi et al., 2010). Prevalence of human brucellosis in Libya was observed to be 40% using RBPT, SAT and ELISA (Ahmed et al., 2010).

In Bangladesh, seroprevalences of brucellosis were 11.11% in veterinary personnel, 6.45% in dairy workers and 4.67% in farmers using RBPT, STAT (Muhammad et al., 2010). Seroprevalence of 9.3% was observed among children by

RBPT and ELISA in Jordan (Al- MAjali and Shorman, 2009). Similarly, prevalence of 21.7% was detected in abattoir workers by IgG ELISA in Lahore district of Pakistan (Mukhtar and Kokab, 2008). In Jordan, true seroprevalence of brucellosis among children was found to be 11.6% by RBPT and ELISA (Al-Majali and shorman, 2009).In Turkey, *Brucella* sero-positivity was detected in 5.4% by STAT while, 11.9% samples were positive by Rose-Bengal test (Vancelik et al., 2008).

Significant titer for brucellosis was observed in 0.66% human serum samples by SAT and ELISA in Gyeongsangbuk-do, Korea (Lee et al. 2007). Seroprevalence rate of 4.8% was reported using RBPT positive and 2-MET as confirmatory test among occupationally exposed persons in Addis Ababa abattoirs (Kassahun et al., 2006). In a study on prevalence of brucellosis in a West Bank Palestinian population revealed 4.4% prevalence among women of child bearing age by ELISA (Karplus et al., 2007). Yacoub et al. (2006) determined prevalence of brucellosis as 29.3% in the suburban semirural area of Basra, Iraq.

A total of 94.1% human sera were positive by SAT for brucellosis and 5.3% were positive for 2-MET in Yazad, Republic of Iran (Salari et al., 2003). Cetinkaya et al. (2005) found 4.8% human serum samples to be positive for brucellosis by RBPT and STAT in western Anatolia, Turkey. Seroprevalence of brucellosis among at-risk group was 7% and 4.1% in normal population by RBPT, SAT, CFT and ELISA in Jordan (Al Ani et al., 2004). Seroprevalence of 3.8% was detected in human serum samples by indirect ELISA in pastoralist community of Chad (Schelling et al., 2003). *Brucella* seropositivity was detected in 3.2% individuals by STAT in Turkey (Sümer et al., 2003). Seroprevalence of 5.2% was detected in PUO patients by SAT in north eastern Nigeria (Baba et al., 2001).

2.4.6 Seroprevalence of human brucellosis in India

Human brucellosis with varying prevalence rates has been reported in India. Kumaraswamy et al. (2015) observed that 15% individual from high risk group were positive for brucellosis by IgG ELISA. Similarly, among the 154 individuals at high-risk screened, *Brucella* antibodies were detected in 9.74% individuals (Aniyappanavar et al., 2013). The seropositivity rate was 30.76% among veterinarians, 14.28% among cattle businessmen, 9.67% among butchers, and 3.79% among animal owners. Anti-*Brucella* antibodies were observed in 17.5% of human sera in Maharashtra (Patil et al., 2013). IgG was raised in five persons, whereas, anti-*Brucella* IgG plus IgM were raised in two persons. Out of these seven persons, five were veterinarians.

Deepthy et al. (2013) reported that 23.3%, 0.83% 5.41% samples from occupationally exposed groups were positive for antibodies to *Brucella abortus* by RBPT, STAT and I-ELISA respectively. Seropositivity among PUO cases was reportd to be 4.79%, 4.41% and 4.41% by RBPT, SAT and 2ME, respectively in Bijapur, Karnataka (Mangalgi et al., 2012). Appannanavar et al. (2012) observed that 9.94% patients with PUO were positive by SAT at a tertiary care center in north India. Overall prevalence of 3.6% was observed among patients with clinical symptoms by indigenously developed indirect ELISA in Karnataka (Agasthya et al., 2012). Shalmali et al. (2012) observed seroprevalence of brucellosis among occupationally exposed human beings as 6.66% using RBPT, STAT, 2-MET, dot-ELISA and indirect-ELISA in Himachal Pradesh.

Positivity for anti-*Brucella* antibodies was 25.72%, 26.66%, 37.14%and 6.00% in PUO patients, meat handlers, veterinarians, milkman and healthy controls, respectively in western Rajasthan (Prakash et al., 2012). Sathyanarayanan et al. (2011) observed 61 seropositive cases by SAT from a tertiary care centre in

Karnataka. Seroprevalence of 2.4% in general population and 11.1% in veterinary staff was reported (Basavaraj et al., 2011).

In an epidemiological survey in Ludhiana, Punjab, Yohannes and Gill (2011) observed 24.5% human sera to be positive by RBPT and diagnosis was established in 26.6% using STAT with a titre range between 80 and 1280 IU/ml. In another study, 4.17% serum samples from farmers in contact with the animals tested positive for brucellosis (Kumar et al., 2010), whereas, seropositivity among patients with PUO was found to be 3.59%. Mantur et al. (2011) identified that 9.9% cases were positive by SAT, 32.7% by Coombs test, while the immunocapture test confirmed the diagnosis in 36% patients. Mantur et al. (2010) observed that 25% cases were positive by SAT whereas ELISA detected brucellosis among 60.9% cases.

Jain and Tilak (2008) observed diagnostic titers ($\geq$160) in 31.25% cases by SAT. Seropositivity was reported among samples from 2.26% 2.26%, 19.69% of high risk group individuals using RBPT, SAT and Indirect ELISA, respectively in Karnataka (Agasthya et al., 2007). Mantur et al. (2006) reported seroprevalence of 1.8% by SAT in Karnataka. From Bikaner, Rajasthan, 175 cases of brucellosiswere reported (Kochar et al., 2007). Bedi et al. (2007) observed seroprevalence of 6.25% among veterinary doctors, 7.9% veterinary pharmacist and 8.8% of class IV employees in Punjab. Kollannur et al. (2007) observed seropositivity of 34.4% among farm personnel. The overall seropositivity for brucellosis in human samples from central Kerala using different serological tests was 1.6% (Ajay Kumar and Nanu, 2005). Mantur et al. (2004) reported that 1.6% children had diagnostic titers for brucellosis in Karnataka.

Mudaliar et al. (2003) reported 5.3% seropositivity among animal handlers in Maharashtra. Seropositivity was observed in 6.8% (Sen et al., 2002) and 0.8% (Kadri

et al., 2000) of the patients with PUO. Human sera tested were positive by dot-ELISA (4.97%) and by RBPT and STAT (1.38% and 0.82%, respectively) (Thakur and Thapliyal, 2002). Among thcellosisese 17.39% positive samples were from field veterinarians and abattoir workers.

Seropositivity for brucellosis was observed to be 25.5% employing dot ELISA among abattoir associated personnel (Barbuddhe et al., 2000). Kumar et al. (1997) detected seroprevalence of 20.60%, 12.75%, 50.30% and 25.45% by RBPT, STAT, CFT and Dot-ELISA, respectively among abattoir personnel in Delhi. In an earlier serological survey of personnel from veterinary hospitalsnd slaughter house of Delhi, seropositivity of 27.7% was detected a (Rana et al., 1985). Among the patients referred to Karnataka Medical College, Hubli, seropositivity for brucellosis was reported in 3% individuals (Mantur, 1988).

Handa et al. (1998) observed 6.6% seropositivity among persons with fever of unknown origin in North India. Positive *Brucella* agglutins were demonstrated in 6.46% of women having a history of spontaneous abortions in Gujarat, India (Panjarathinam, 1984). Dairy personnel in contact with infected animals were seropositive (8.5%) for brucellosis (Mathur, 1964).

2.4.7 Isolation and identification

2.4.7.1 Isolation and identification of *Brucella* from bovines

Thirty-seven *B. abortus* biovars 1 and 3 isolates were obtained from samples collected from a cattle herd, which implemented vaccination using RB51 and test-and-slaughter policies (Dorneles et al., 2014a). *Brucella abortus* strains were isolated from 86.8% of animals on combined culture of lymph nodes (O'Grady et al., 2014). Individually, the highest isolation rate was from the retropharyngeal lymph node. Sola et al. (2014) observed that 28.3% samples consisting of lesions and viscera

of slaughtered cattle were positive by real time PCR. An isolate exhibiting typical characteristics of *B. suis* biovar 2 and confirmed by real-time PCR was recovered from cattle uterus (Fretin et al., 2013). *Brucella* spp. was recovered from 7.5% blood and milk samples collected, and 22.4% samples were found positive by *IS711* PCR in Sudan(Abdalla and Hamid, 2012). In an another study, Buyukcangaz et al. (2011) isolated *Brucella* spp. from 31.9% organ homogenates of aborted fetuses and out of these 21.1% were from cattle. PCR amplification of *Brucella*-DNA was observed in 55.76% bovine milk samples and Brucella organisms were isolated from 46.15% samples of cows' milk by direct culture method (Hamdy and Amin, 2002).

A total of 30 *B. abortus* biovar 1 isolates were recovered from milk (n=5), aborted fetuses (n=13), and vaginal swabs (n=12) mostly from cattle in Pakistan (Ali et al., 2014). Buyukcangaz and Sen (2007) obtained 8 *Brucella* isolates from aborted bovine fetuses in Turkey. Langoni et al. (2000) reported prevalence of *B. abortus* in 30.61% milk samples by culture isolation in Brazil. Of these 2.04% isolates were belonging to biotype 1, 16.32% of biotype 2 and 12.25% were biotype 3. A total of 397 isolates of *Brucella* were isolated by culturing MRT positive milk samples (Zowghi et al., 1990) The isolates belonged to *B. abortus* biotype 2, 3 and 9. Ewalt (1989) isolated *B. abortus* from 52.3% samples of supra mammary lymph nodes of cattle.

2.4.7.2 Isolation and identification from bovines in India

Isolation and identification of the organism from clinical samples is a gold standard for diagnosis. Abortion storms in cattle and buffalo were investigated and 25 *B. abortus* isolates were obtained from morbid materials (Chand and Chhabra, 2013). Isolation of pathogen from the farms on which abortions had occurred indicated that disease was actively circulating among animals. Ghodsara and Roy (2012) obtained

10 *Brucella* isolates from cases of abortion and retention of placenta in cows, buffaloes, does and bitches in Gujrat state. Nagalingam et al. (2012) reported that 28 field isolates of *B. abortus* from Karnataka were belonging to either biotype 1, 2 or 4 by AMOS PCR.

Isolation of *Brucella* spp. was reported from 2 of the 22 milk samples (Trangadia et al., 2010) and 5 out of 10 isolates were confirmed as *B. abortus* by using *B. abortus* specific *bcsp31* and omp2a/2b primers (Ghodsara et al., 2010). Kaur et al. (2006) obtained 17 isolates of *B. abortus* from aborted materials from bovines, of them 11 belonged to biotype 1, 3 to biotype 3, and 3 belonged to biotype 2.

2.4.7.3 Isolation and identification from humans

A gene encoding the 31-kDa *B. abortus* antigen was amplified in 73.8% of the tested sera and 63.8% samples demonstrated amplification of 3'-end of IS711 using *B. abortus* specific primers (Garshasbi et al., 2014). Also an amplification of *B. melitensis*-specific primers was observed in 4.4% of the tested samples using PCR. Hekmatimoghaddam et al. (2013) reported that blood culture was successful in 5% cases and 20.8% samples demonstrated amplification by PCR.

Escobar et al. (2013) isolated six *B. suis* strains from blood cultures of workers in a pig slaughterhouse. Of these three were belonging biovar 1 and three with atypical characteristics. Employing multiplex PCR, 23% samples were detected positive for *B. abortus* and 25% for *B. melitensis* (Mirnejad et al., 2013).

Brucellosis cases were diagnosed in 15.9 % cases using a combination of blood culture and PCR in Saudi Arabia (Asaad et al., 2012). Nimri (2003) detected Brucellosis was detected in 120 cases (72.7%) by genus specific PCR, all of the cases were seropositive and the organisms were isolated from 20 cases. .

2.4.7.4 Isolation and identification from humans in India

Comparison of lysis concentration (LC), clot culture and conventional Castaneda blood culture techniques for the isolation rate and recovery time in the diagnosis of human brucellosis revealed overall blood culture positivity to be 24.8%, 43.1% and 34.9% by conventional, LC and clot culture techniques, respectively (Mangalgi and Sajjan, 2014). It was observed that LC and clot culture techniques were better than the conventional technique for the blood culture. A case of brucellosis caused by *B. suis* which was negative for *Brucella* IgM ELISA but positive in blood and bone marrow cultures was diagnosed (Naha et al., 2012).*B. melitensis* was recovered from a *Brucella* endocarditis case by blood culture and confirmed by the *omp*2 PCR (Pawar et al., 2011). A meningitis case in a 11-month-old infant transmitted by breast feding was reported (Tikare et al., 2008). *B. melitensis* was isolated from breast milk. Mantur et al. (2007) observed that culture of blood clots was more sensitive for isolation of *Brucella* than whole blood culture. Mantur et al. (2006) isolated *B. melitensis* from 191 (55.3 %) of the 345 cases using blood culture technique. Cutaneous manifestation due to *Brucella melitensis* was confirmed by *IS711* PCR (Mutnal et al., 2007).

Vajramani et al. (2005) isolated *B. melitensis* from intra-medullary spinal cord abscess. *B. melitensis* was isolated from 43 pediatric patients over a period of 13 years (Mantur et al., 2004). Deepak et al. (2003) isolated *Brucella* from bone marrow aspirations. The small ruminants were confirmed as the sources of transmission of human brucellosis in Haryana (Mathur, 1968). *B. melitensis* was isolated from human blood and milk samples from goats and sheep.

2.4.8 Molecular subtyping methods

Molecular typing methods are useful in tracing back the sources of transmission in epidemiological settings (Rothman and Greenland, 1998). This helps

in planning and implementation of appropriate counter measures in a time bound way and for prevention of future occurrence of the infection. Appropriate subtyping methods are, therefore, vital for planning prevention and control of brucellosis. *Brucella* spp has high degree of genetic homogeneity (>90%) as shown by DNA–DNA hybridization, therefore, it has been challenging to discriminate among different species employing molecular techniques (Verger et al., 1985).

A variety of techniques are available for molecular subtyping of bacteria including *Brucella.* The techniques which can be used are AP-PCR (Fekete et al., 1992), ERIC (enterobacterial repetitive intergenic consensus sequence), repetitive intergenic palindromic sequence-PCR (Mercier et al., 1996, Tscherneva et al., 1996), RAPD (random amplified polymorphic DNA) (Huber et al., 2009; Tscherneva et al., 2000), PCR-RFLP (PCR-restriction fragment length polymorphism) (Dawson et al., 2008; Al Dahouk et al., 2005), amplified fragment length polymorphism (Whatmore et al., 2005), SNP (Marianelli et al.,2006; Scott et al., 2007) and multi locus sequence typing (Whatmore et al., 2007).

Variable number tandem repeats, used frequently for DNA typing in forensic applications has been suggested to be highly discriminatory for *Brucella* (Al Dahouk et al., 2007; Le Fleche et al., 2006;). Among others, multiple locus variable number tandem repeats analysis (MLVA) has been showed to differentiate among unrelated *Brucella* isolates which are difficult to differentiate by conventional microbiological methods. For *Brucella,* MLVA-21, MLVA-16 and MLVA-15 loci panels have been described (Al Dahouk et al., 2007; Bricker et al., 2003). Based on the MLVA-16 scheme, a collaborative public online database was established for creation of a global epidemiological map of *Brucella* (http://mlva.u-psud.fr/brucella/).

Investigation of the genetic stability of *B. abortus* strains isolated from an outbreak by MLVA-16 demonstrated lower genetic diversity among *B. abortus* strains (Dorneles et al., 2014a).. Geographic clustering of some genotypes among *B. abortus* isolates was shown MLVA16 panel 1 and 2 (Minharro et al., 2013). Similarly, genotyping of *B. ovis* strains by MLVA16 revealed thirteen distinct genotypes with a Hunter-Gaston diversity index (HGDI) of 0.989 among 14 *B. ovis* strains (Dorneles et al., 2014b). High discriminatory power (HGDI 0.972 and 0.902) was shown by MLVA16 analysis for both *B. abortus* and *B. melitensis*. Markers from panel 1 and 2A demonstrated higher diversity in *B. abortus* as compared to *B. melitensis* isolates (Ferreira et al., 2012).

2.4.8.1 Biotype identification

Identification of species and biotypes is important epidemiologically as soon as the culture is confirmed as *Brucella*. This can be accomplished in specialized or reference laboratories. The biotyping schemes are laborious and include a battery of tests including carbon dioxide requirement (CO_2), hydrogen sulphide (H_2S) production, dye sensitivity, phage lysis and testing of monospecific A, M or R specific antisera for agglutination (Poester et al., 2010).

Table 2.1 Differential characteristics of the biotypes among *Brucella* spp.

Species	**Biotype**	**CO_2 requirement**	**H_2S production**	**Growth on dyes**		**Agglutination with antisera**		
				Thionin	**Basic fuchin**	**A**	**M**	**R**
B. abortus	1	(+)	+	-	+	+	-	-
B. abortus	2	(+)	+	-	-	+	-	-

B. abortus	3	(+)	+	+	+	+	-	-
B. abortus	4	(+)	+	-	+	-	+	-
B. abortus	5	-	-	+	+	-	-	-
B. abortus	6	-	-	+	+	+	-	-
B. abortus	9	-	+	+	+	-	+	-
B. melitensis	1	-	-	+	+	-	+	-
B. melitensis	2	-	-	+	+	+	-	-
B. melitensis	3	-	-	+	+	+	+	-
B. suis	1	-	+	+	-	+	-	-
B. suis	2	-	-	+	-	+	-	-
B. suis	3	-	-	+	-	+	-	-
B. suis	4	-	-	+	-	+	+	-
B. suis	5	-	-	+	-	-	+	-

Keys +: Positive, -: Negative, (+): Usually positive on primary isolation

Thirty *B. abortus* strains were biotyped as biovar 1 in Pakistan (Ali et al., 2014), while, 73 and two strains were typed as *B. melitensis* biovar 3 and as *B. abortus* biovar 3, respectively in Turkey employing conventional biotyping methods Parlak et al., 2013). Eight isolates were confirmed as B. *abortus* biovar 3 among cattle herds in Srilanka (Priyantha, 2011). Cerekci et al. (2011) identified 187 *B. melitensis* biovar 3 isolates of human origin using conventional methods. In another study, 9 *B. abortus* isolates obtained from domesticated elk in Korea were belonging to biotype 1 by conventional techniques (Buyukcangaz and Sen 2007; Her et al., 2010). Characterization of *Brucella* spp. isolates from aborted bovine fetuses revealed 7 isolates as *B. abortus* biotype 3 and one as *B. melitensis* biotype 3. *B. abortus* biovars

1-4 and 6 were described by classical biotyping and PCR in Brazil (Minharro et al., 2013).

2.4.8.2 Virulence profiling

Little data is available about detection of virulence genes from *Brucella* spp. Derakhshandeh et al. (2013) observed that frequency of the virulence genes, the *bvfA*, *virB,* and *ure* among 42 *Brucella melitensis* isolates was 78.50%, 73.80%, and 88.09%, respectively.

2.4.8.3 PFGE

Pulsed field gel electrophoresis (PFGE) has been used as molecular typing technique in epidemiological investigations worldwide owing to its high discriminative power. In this technique large DNA restriction fragments are migrated variably in an alternating electrical field (Nassonova, 2008). By comparing the fingerprints of the isolates, their clonality can be studied. The adoption of PFGE was facilitated by its use in international surveillance networks, such as PulseNet (CDC, USA) and standardization of protocols for important food-borne pathogens (*E. coli*, *Listeria*, *Campylobacter*). In spite of availability of recent typing methods, PFGE is considered as the gold standard in a number of national and international surveillance programmes. *Brucella* species were analysed by PFGE and its usefulness for distinguishing between species of *Brucella* has been shown (Allardet-Servent et al., 1988; Jensen et al., 1995; Jensen et al., 1999). Use of low-frequency cleavage enzymes gives better discrimination between *Brucella* species by PFGE (Jensen et al., 1999).

PFGE is a major epidemiological tool for differentiating pathogenic bacteria but has limited application for routine typing of *Brucella* isolates due to limited genetic diversity at sub species level observed in genus *Brucella* (Swaminathan et al.,

2001). Previously, minor variants of *B. ovis* (Ridler et al., 2005), discriminating between a vaccine and field strains (Jensen et al., 1995) and differentiating isolates from porpoises, seals and dolphins (Jensen et al., 1999; Bourg et al., 2007) has been performed using PFGE. Brower et al. (2007) compared techniques for differentiation of *B. canis* strains isolated from outbreaks in kennels from Wisconsin, USA in the situations of the dog trade observed that all of the isolates were genetically homogenous and as such undistinguishable by ribotyping, PFGE and OMPA. Li et al. (2013) observed 6 PFGE clusters within 32 *B. suis* isolates recovered in China.

2.5 Risk Factors

2.5.1 Risk factors for bovines

The most crucial risk factor which facilitated intra-herd spreading of brucellosis was found to be abortion or delivery of an infected animal on the farm (Chand and Chhabra, 2013). Seropositivity for brucellosis in cattle was associated with age of the animal at the animal population level in Niger (Boukary et al., 2013). Mohammed et al. (2011) observed that age, sex, location, and herd size played role in the epidemiology of brucellosis among cattle herds in Jigawa state, northwestern Nigeria. Seropositivity was observed in herds having a history of abortions. Smaller herds with one to two cattle were less seropositive as compared to large herds with more than eight cattle in Tajikistan (Lindahl et al., 2014) and the number of calvings per cow was associated with seropositivity. Sanogo et al. (2012) reported that brucellosis seropositivity was associated with age of animal and herd size in Ivory Coast. The major risk factors for brucellosis in Uganda were larger herd size and a history of abortion (Makita et al., 2011).

2.5.2 Risk factors for humans

Handling a case of abortion in animal and close proximity to neighborhoods was observed to be the major risk factors for brucellosis in humans in Tanzania (John et al., 2010). Also, brucellosis had a significant association with untreated milk consumption, slaughtered meat, and direct contact with animals and individuals who had a nomadic lifestyle in Iran (Alavi et al., 2014). Rahman et al. (2012) observed that contact with livestock was an important risk factor for *Brucella* infection of individuals in high-risk group in Bangladesh. Exposure to animals at home, handling aborted animals, slaughtering/butchering, and processing raw milk were associated significantly with the risk for brucellosis in Uzbekistan (Earhart et al., 2009) and number of deliveries handled and injury during *Brucella* vaccine administration were risk factors for occupational brucellosis among veterinarians in Turkey (Kutlu et al., 2014). Close contact with livestock, eating of fetus and placenta were the major risk factors for human brucellosis in Ecuador (Ron-Román et al., 2014). Milking sheep, consumption of raw feta cheese made from sheep and goat milk, consumption of milk of cow and boiled feta cheese were risk factors for human brucellosis in Jordan (Abo-Shehada and Abu-Halaweh, 2013). While, cutting animal throats and cleaning slaughtered animal parts were the major risk factors among slaughterhouse workers in Tanzania (Swai and Schoonman, 2009).

2.6 Prevention and Control

Currently, no vaccine is available for human brucellosis. Prompt reporting of the disease to health authorities and status of prevalence are important to prioritize and plan control policy (Smits and Kadri, 2005). Awareness about effective control measures among the patients and knowledge of disease among the doctors and health workers are essential for effective prevention of the disease. Milk is the principal sournce of transmission from infected animals. Boiling of milk and proper

pasteurization of dairy products can help to prevent from infection. Awareness among the occupational groups such as farmers, shepherds, and veterinarians is essential to prevent the risk of exposure. Importanance of vaccination of animals should be emphasized.

Vaccination of animals with available vaccine is relatively cheap and effective (Smits and Kadri, 2005). Wearing of protective clothing while handling suspected animals and while working with aborted animals will help to avoid infection (Corbel, 2006).

Brucellae can be used as weapons for bioterrorism. Airborne brucellae can be prevented by the M40 mask during biological attack event. In case of accidental exposure of live organisms, decontamination of clothing, skin, and surfaces with standard disinfectants can help to minimize risk of infection (Dembek and Zygmunt, 2007).

Besides vaccination of animals, control of movement of animals, mass testing, segregation of infected animals are effective control of brucellosis among animal population (Corbel, 2006). Test and slaughter policy can be implementaed considering the, economic and prevalence factors. This can be applied if the herd prevalence is very low (e.g. 2%) (Corbel, 2006).

Clinical disease can be eliminated by vaccination of animals. Reduction of excretion of organisms in natural secretions can also be achieved by vaccination (Meeusen et al., 2007). Currently, *B. abortus* strain 19 for bovines and the attenuated *B. melitensis* strain Rev.1 vaccine for sheep and goats have proved to be superior (Adone et al., 2005; Corbel, 2006). The rough *B. abortus* strain RB51 vaccine has been used with success in the developed countries (Mantur and Amarnath, 2008).

2.7 Treatment

Treatment of infected cattle or pigs is not recommended. However, infected dogs can be treated with long-term antibiotic regime with variable success. Rams have been treated with antibiotics successfully; however, it is usually uneconomical (Spickler, 2009). Being facultative intracellular, brucellae are inaccessible to antibiotics. Though, a number of antimicrobials are effective against *Brucella* species; their clinical efficacy differs with *in vitro* susceptibility (Hall, 1990). Treatment of acute brucellosis in adults with rifampicin 600 to 900 mg in combination with doxycycline 200 mg twice daily has been recommended by the World Health Organization (FAO/WHO 1986) and the treatment has to be continued for a minimum period of six weeks. Streptomycin (1 g/day) intramuscularly for 2-3 weeks in combination with tetracycline (2 g/day) orally for 6 weeks has been reported to give lesser relapses (Ariza et al., 1985; Mantur et al., 2006). Trimethoprim-sulfamethoxazole (TMP/SMX) is also recommended in triple regimens. Ciprofloxacin and ofloxacin in various combinations have been tried clinically (Karabay et al., 2004).

Alternative combinations of drugs for treatment of brucellosis are being explored. Brucellosis in children can be treated with an oral administration of a combination ofrifampicin 10 mg/kg and doxycycline 4 mg /kg daily for six weeks. To prevent relapse, concomitant intramuscular administration of gentamicin (5 mg/kg/day) for the initial period of 5- 7 days of therapy has been advised (Hall 1990; Mantur et al., 2004).

For treatment of brucellosis during pregnancy, rifampicin with or without a combination of TMP/SMX has been suggested. Complications due to brucellosis can be treated adequately with longer courses of standard regimens. Neurobrucellosis is difficult to treat. Therapy with a combination of two or three drugs (doxycycline,

rifampicin, and TMP/SMX) has been recommended (McLean et al., 1992). In case of complications such as brucellar endocarditis, doxycycline, rifampicin and trimethoprim-sulfamethoxazole in combination can be advised; however, surgical interventions (valve replacement) with antibiotic treatment have been suggested (Mantur and Amarnath, 2008).

Chapter 3
Determination of seroprevalence and identification of risk factors associated with brucellosis among humans and animals in Goa region

Introduction

Brucella infections in humans and animals cause serious and debilitating conditions. In humans, the disease causes severe morbidity and complications which are often misdiagnosed or under diagnosed. The disease is difficult to diagnose due to overlapping clinical presentation, lack of diagnostic facilities, lack of awareness about symptoms and transmission routes. Bovine brucellosis caused by *B. abortus* causes economic losses in dairy farming. Infections of female animals result in abortion in advance pregnancy, birth of dead calves, retention of placenta, metritis, infertility, repeat breeding, and reduction of milk yield. The aborted animal becomes a carrier of the disease with shedding of *B. abortus* organisms in large amounts through placenta, fetus and uterine discharges, and in milk (Radostits et al., 2007). Also, human brucellosis severely affects health of the individual affecting the general well being which results in loss of man days.

Brucellosis has been reported worldwide, however, its incidence is more in countries without standardized and effective public health and animal health programmes. India is agriculture dependant country and animal husbandry forms the backbone of rural economy. Government is promoting dairy farming through various policies. Brucellosis is significant threat to dairy animals. Trading of animals within different regions is responsible for spreading the disease to new locations. Test and slaughter of a *Brucella* positive animal is not possible in India due to religious aspects. Therefore, complete eradication of *Brucella* is a challenging task for India.

In India, brucellosis had been reported first in the previous century and its presence has been reported countrywide (Renukaradhya et al., 2002). In India, epidemiologically, brucellosis has been reported in different species of farm animals such as cattle, goats, sheep, buffaloes, horses, yaks, camels and pigs. Because of close

proximity of farmers in rural areas with their animals and high rate of consumption of unpasteurized dairy products, human brucellosis is common in rural areas. Vending of unpasteurized dairy products in urban areas may contribute to its transmission. The long term serological studies at national level indicated that about 5% cattle and 3% buffalos could be infected with brucellosis (Renukaradhya et al., 2002). Many authors have reported different seroprevalence rates in PUO patients, veterinarians, animal handlers, slaughterhouse workers as well as other occupationally exposed individuals.

Serological evidences state that brucellosis is endemic in India. However, true prevalence estimates are lacking due to absence of data in some regions. Serological tests are accurate methods of diagnosis when used in combinations. Different agglutination based assays e. g. RBPT, STAT, ELISA, CFT are useful for quick and accurate diagnosis of brucellosis. RBPT is a primary screening test for both human and animal samples. For diagnosing human brucellosis, STAT is useful whereas, it is regarded unsatisfactory for bovine samples (OIE, 2009). ELISA had proved useful for diagnosis with high specificity. Sensitivity of these tests can be increased by use of certain reagents which helps to reduce the cost.

Various risk factors such as use of semen from infected bulls for artificial insemination and poor hygiene at farm are attributed to the dessimination of the infection (Smits and Kadri, 2005). Different animal husbandry practices, unrestricted trade and movement of animals, environmental conditions, intensive dairy farming are major risk factors responsible for maintenance of brucellosis in any given geographical area (Crawford et al., 1990). A study conducted in Punjab revealed that brucellosis in animals was infrequent in villages where farmers provided adequate floor space, proper ventilation, and sunlight to animals (Saini et al., 1992). In India, increasing demand for foods of animal origin (milk and meat), intensive animal

rearing practices and rapid movement of animals have been attributed to higher occurrence of the disease (Chand and Chhanra, 2013).

Human brucellosis has been regarded as public health problem worldwide. It has been responsible for serious health problems and economic losses in countries affected. Consumption of unpasteurized milk and milk products and other foods is a major risk factor for humans. The disease is mainly transmitted through animal secretions. Thus, handling animals, assistance during calving increase chances of getting infected. Lack of awareness in clinicians and general public mostly contribute to under-diagnosis (Thakur et al., 2002). Risk groups include veterinarians, abattoir associated personnels, farm workers, hunters and laboratory personnel handling live cultures. Goa state has many organized farms and farm workers live in close contact with farm animals. Large scale seroprevalence survey in Goa has not been performed. Therefore, the status of disease in human and animal population is unknown. Thus, it is important to study the prevalence of the disease and the risk factors for transmission of brucellosis in human and animal populations of Goa.

3.1 Collection of samples

3.1.1 Human

Human blood samples (n=282) from cases of pyrexia of unknown origin (PUO) (n=243) and occupationally exposed individuals (n=39) were collected from individuals refered to Goa Medical College and Hospital, and private hospitals with prior consent of patients. Blood samples were centrifuged at 3,000 g for 10 min for separation of serum and stored at -20^{0}C till further use.

3.1.2 Animal

Blood samples (n=355) were collected from 12 different dairy farms in Goa. The farms were selected on basis of incidences of abortions reported to state veterinary officials. The samples were collected from aborted animals, animals with reproductive disorders as well as in contact healthy animals. Ten milliliters of blood was withdrawn from jugular vein of cattle and collected aseptically in sterile plain tubes. Serum samples were separated from clotted blood samples as describe in section 3.1.1 and stored at -20^{0}C till further use.

3.2 Serological tests

3.2.1 RBPT

The RBPT antigen manufactured at Division of Biological Products, Indian Veterinary Research Institute (IVRI), Izatnagar, India was procured for the test. The test was performed as described by Alton et al. (1975). A total of 100 µl serum was placed on a clean glass slide and equal quantity of RBPT antigen was mixed with serum and the slide was slightly shaken for uniform mixing. The reaction was allowed at room temperature for 3 min and observed for agglutination. The formation of clear clumps was considered a positive test while the absence of clear clumps was considered negative.

3.2.2 STAT

The method recommended by OIE was followed. Phenol killed *B. abortus* S99 plain antigen procured from IVRI, Izatnagar, India was used for the test. Eleven agglutination tubes were placed in a rack. 0.8ml of 0.5% phenol saline was added to the first tube of the series. 0.5ml of phenol saline was added to all the other tubes except in tube no. 9, 10, and 11 which contained 1.25, 1.50 and 1.75ml of phenol saline, respectively. After adding 0.2 ml serum to the first tube and the contents were mixed. 0.5 ml of this mixture was transferred to the second tube. This process was repeated till the 8^{th} tube, there after 0.5ml of the content was discarded. 0.5 ml of the plain antigen was then added to tubes 1 to 8, giving a final dilution of 1:10, 1:20, and 1:40 and so on.

Antigen, 0.75, 0.50 and 0.25 ml, respectively was added to the tubes 9, 10, and 11 which were kept as controls. These tubes were incubated for 24 hours at 37°C and the results were read. The results were compared with the antigen control tube showing 50% agglutination. The highest dilution showing 50% agglutination was taken as the titer. The titers of ≥1:80 and ≥1:160 were considered positive for cattle and human samples, respectively.

3.2.3 Indirect ELISA

3.2.3.1 Indirect ELISA for bovine brucellosis

For diagnosing bovine brucellosis, Protein G based indirect ELISA kit developed by National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI, formerly PD_ADMAS), Hebbal, Bengaluru, India was used and the test was performed as instructed by the manufacturer (Shome et al., 2014). The controls and samples were tested in duplicates. Absorbance at 492 nm was measured by

ELISA reader (Thermo Scientific, USA). The mean absorbance values were determined. Percent positivity (PP) was determined according to formula provided by the manufacturer. Samples showing PP values ranged between 55% to 65% were considered as moderate positive and values more than 65% as strong positive.

3.2.3.2 Indirect ELISA for human brucellosis

Indirect ELISA for human brucellosis was carried out as illustrated by Agasthya et al. (2012). The *Brucella abortus* S99 smooth lipopolysaccharide (S-LPS) antigen and the Positive serum showing higher absorbance in ELISA were procured from IVRI, Izatnagar. The sera taken from apparently healthy individuals were employed as the negative control. Positive serum was diluted in negative serum (1:20) to prepare moderate positive control. Checkerboard titration was used to establish working dilutions of S-LPS antigen, control serum samples and rabbit anti-human HRP conjugate (Sigma). The substrate, o-phenylenediamine dihydrochloride (OPD), bovine gelatin (Sigma) and 96-well ELISA plate (Nunc polysorp) were used. Control and test sera were tested at 1:100 dilutions. Sera were considered positive with positive to negative ratio of $\geq$3.

3.2.4 IgG and IgM ELISA for human brucellosis

The diagnostic kits were procured from Nova Tec Immunodiagnostica GmbH and the test was carried out as per manufacturer's instructions. The mean absorbance value of cut-off controls and patient's samples was determined. The results were expressed in Nova Tec-Units (NTU) with a positive titer of 11 NTU or more as positive as per manufacturer's guidelines.

3.3 Identification of risk factors

Risk factors for brucellosis among cattle and human population in Goa were identified through field visits. A questionnaire providing epidemiological data was

prepared. A survey for identification of risk factors was conducted on 11 cattle farms having seropositive cases. Seroprevalence was calculated as number of sample positive for both RBPT and ELISA divided by total number of samples. Presence of risk factors was correlated with seropositivity. OR (Odds ratio) for all the variables was calculated using PAST software (version 3.01). Similarly in case of human samples, a questionnaire providing clinical history and related information of patients was prepared. The information of patient was obtained from concerned medical practitioner or head of the medical department. Positive reaction, in any, of the serological test (RBPT/STAT/Indirect ELISA/IgG ELISA) was regarded as seropositive case. P values less than 0.5 was regarded as significant.

3.4 Results

3.4.1 Seroprevalence among humans

Sera samples were tested by employing a battery of tests. Twenty five (8.86%) samples were positive to at least one test used (Table 3.1). Of the samples tested, RBPT, indirect ELISA and IgG ELISA detected 4.25%, 6.02% and 4.96% samples as positive, respectively. None of the sample demonstrated the presence of IgM titers. Variable STAT titers were demonstrated by RBPT positive samples with diagnostic titers in 10 (3.54%) samples. Ten (25.64%) RBPT positive samples were collected from the individuals with occupational exposure such as animal handlers, dairy workers and paravets. Surprisingly, one serum sample from a frequent international traveller to brucellosis endemic region suffering from PUO was found to be positive by RBPT. The individual had no history of animal contact.

Fourteen (35.89%) samples from the occupationally exposed individuals and 3 (7.69%) from PUO cases were positive by indirect ELISA. On the other hand, 11

(28.20%) individuals exposed occupationally and 3 PUO cases demonstrated positivity by IgG ELISA.

3.4.2 Seroprevalence among animals

Of the 355 cattle sera, 98 (27.60%) were positive by RBPT and 106 (29.85%) were positive by STAT (Table 3.2). A total of 123 (34.64%) sera were positive by Indirect ELISA, of these 105 samples were strongly positive for the test and 18 were moderate positive (Table 3.3).

3.4.3 Identification of risk factors

It was observed that awareness about brucellosis was lacking in most of the farms (OR=8.739, P=0.138) which in turn was responsible for high seroprevalence. Floor space was found to be significant risk factor (OR=0.278, P=0.128). Herd size (OR=1.716) and grazing the animals (OR= 2.197), were also found to be significant risk factors (Table 3.4). In case of human brucellosis, none of the risk factor demonstrated significant odds ratio (Table 3.5), but age of the patient and knowledge about brucellosis were found related to seropositivity. Patients lacking knowledge about brucellosis and patients having age more than 40 years were found more susceptible for disease.

Table 3.1 Results of serological and cultural tests performed on human samples

Case no.	Tests employed					
	Case Type	**RBPT**	**STAT**	**Indirect ELISA**	**IgG ELISA**	**Blood Culture**
1	Farmer	-	1:80	-	-	ND
2	PUO	-	1:80	++	-	ND
3	Farm worker	++	1:160	++	-	-
4	Farm worker	-	1:80	++	-	ND
5	Farm worker	-	1:80	++	-	ND
6	Farm worker	-	1:160	++	-	-
7	PUO	-	1:80	++	-	ND
8	PUO	-	1:80	++	++	ND
9	PUO	-	1:80	-	-	ND
10	Farmer	++	1:160	++	-	-
11	Farmer	++	1:160	++	++	-
12	Farmer	++	1:160	++	++	-
13	PUO	++	1:160	-	++	-
14	Farm worker	++	1:160	++	++	-
15	Farm worker	-	1:80	++	++	ND
16	PUO	-	1:80	-	++	ND
17	Butcher	++	1:160	++	++	-
18	Farm worker	++	1:160	++	++	-
19	Farm worker	-	1:80	++	++	ND
20	Farmer	++	1:80	-	++	ND
21	Farmer	-	1:80	++	++	ND
22	Butcher	++	1:160	++	++	ND
23	Farmer	-	1:80	-	++	ND
24*	Farmer	++	1:80	-	-	++
25	Travel Associated (PUO)	++	1:80	-	-	ND

Keys: - : Negative, ++: Positive, PUO: Cases of pyrexia of unknown origin, ND: Not done, *: culture positive case

Table 3.2 STAT titers of cattle serum samples

STAT titre	Number of samples
1:10	89
1: 20	57
1:40	103
1:80	45
1:160	23
1:320	26
1:640	8
1:1280	4

Table 3.3 Detection of brucellosis among cattle by serological tests

Farm	No. of samples	RBPT positive	STAT positive	ELISA positive	RBPT and ELISA positive
Farm1	39	5	12	18	4
Farm 2	11	3	5	5	1
Farm 3	39	13	10	13	9
Farm 4	40	17	16	23	14
Farm 5	25	3	5	2	2
Farm 6	23	0	1	0	0
Farm 7	25	7	5	3	2
Farm 8	15	9	13	9	8
Farm 9	20	1	3	5	0
Farm 10	53	17	14	17	17
Farm 11	40	21	19	22	18
Farm 12	25	2	3	6	2
Total	355	98	106	123	77

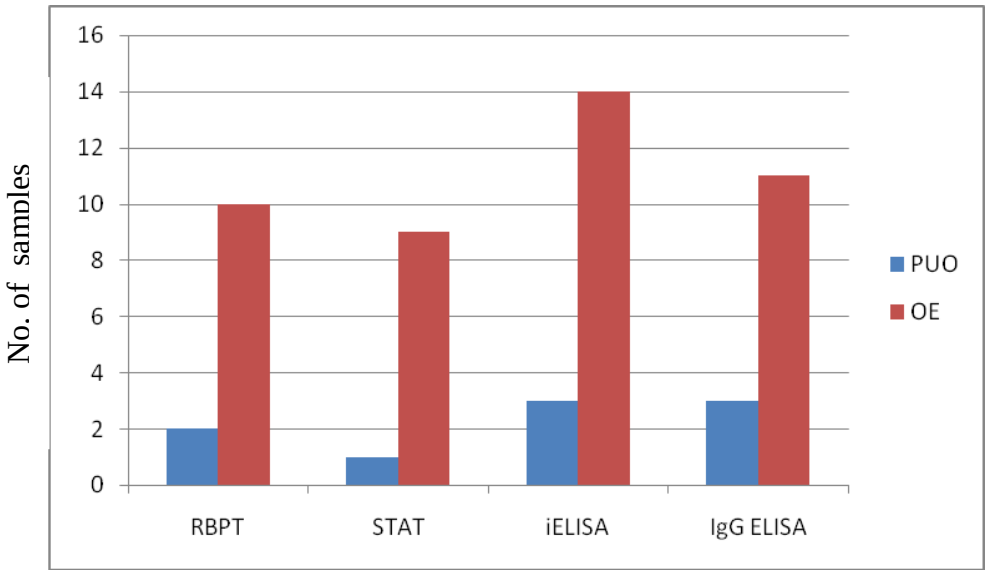


Fig. 3.1 Comparison of different serological tests for detection of brucellosis in humans among cases of pyrexia of unknown origin (PUO) and individuals with occupational exposure (OE) .

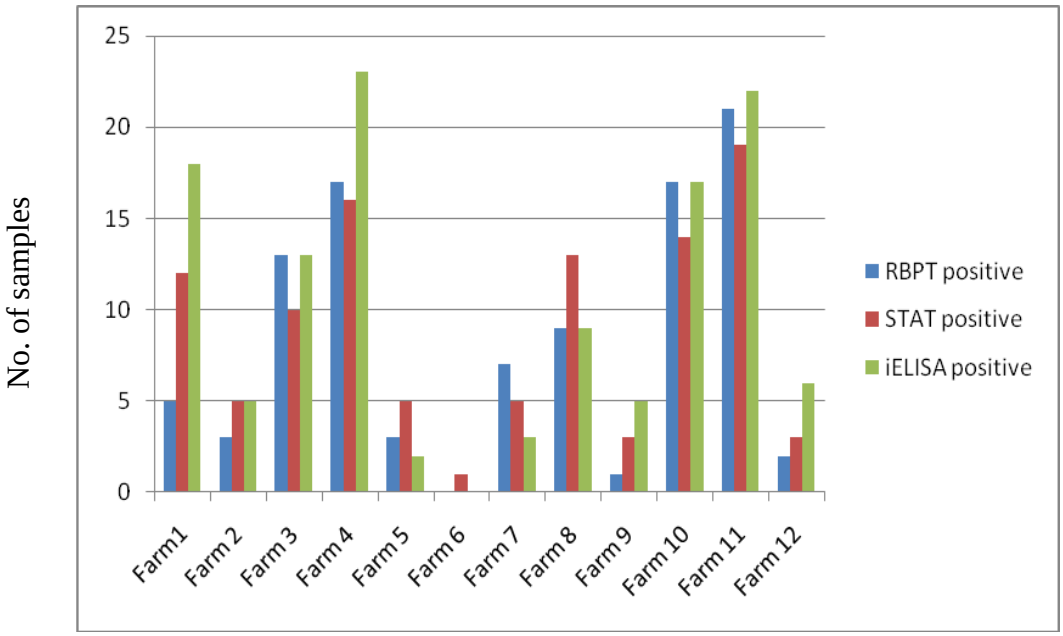


Fig. 3.2 Farm wise seroprevalence of brucellosis among cattle in Goa region

Table 3.4 Risk factors for brucellosis among cattle population in Goa

Risk Factor	Variable	No. of farms	Seropositivity (%)	OR	95% CI	P value
Herd size	< 20	4	6.75	1.716	0.38-7.7	0.4821
	>20	7	20.27			
Breed	Local	2	40.90	0.120	0.00-0.60	0.610
	Cross	9	22.26			
Abortions on the farm	Present	8	26.01	1.035	0.22-4.7	0.963
	Absent	3	1.01			
Floor space	Adequate	4	3.71	0.278.	0.05-1.455	0.128
	Inadequate	7	23.31			
Grazing	Present	5	7.43	2.197	0.512-9.429	0.289
	Absent	6	19.59			
Farm hygiene	Proper	5	13.83	0.793	0.194-3.239	0.7472
	Improper	6	13.17			
Awareness about brucellosis	Present	2	0.67	8.739	0.496-153.9	0.138
	Absent	9	26.35			
Use of milking machines	Yes	3	6.75	1.126	0.2303-5.507	0.883
	No	8	20.27			
Purchase of unscreened animals	Yes	10	26.68	0.123	0.00-6.76	0.305
	No	1	0.33			
Separation of pregnant animal	Yes	2	4.05	1.260	0.196-8.105	0.8074
	No	9	22.97			

Table 3.5 Risk factors for brucellosis among humans in Goa

Risk factor	Variable	No. of cases	Seropositivity (%)	OR	95% CI	P value
Consumption of unpasteurized food products	Yes	8	32	1	0.3908-2.5591	1
	No	17	68			
Occupational exposure	Yes	10	40	1	0.4088-2.446	1
	No	15	60			
Frequent exposure to livestock	Yes	14	56	1	0.4136-2.418	1
	No	11	44			
Age	20-40	6	24	1	0.3584-2.79	1
	40-60	19	76			
Sex	Male	12	48	1	0.4159-2.404	1
	Female	13	52			
Family history	Yes	4	16	1	0.3026-3.305	1
	No	21	84			
Knowledge about brucellosis	Yes	9	36	1	0.4013-2.492	1
	No	16	64			

3.5 Discussion

3.5.1 Seroprevalence and risk factors for humans

Human brucellosis can be diagnosed by isolation of the pathogen from clinical samples, using different serological tests for the detection of antibodies against *Brucella* and also by using the DNA based methods (Al Dahouk et al., 2013). However, the causative agent needs to be diagnosed using a combination of any of these tools for its reliable detection. (Al Dahouk et al., 2013). Moreover, the tests are often difficult to interpret in endemic areas because of continuous exposure of the large part of the population with animals or foods of animal origin that could develop anti- *Brucella* antibodies.

Animal husbandry is a major source of income in rural India. Lack of awareness about farm hygiene and zoonotic infections are increasing the risk of

acquiring infections in human population. In the Indian subcontinent, brucellosis is highly endemic among all livestock species and humans. Brucellosis in humans may be acquired through secretions of infected animals. In humans, the disease needs to be differentiated from tuberculosis, malaria or typhoid fever. The cases of brucellosis may go unrecognized, undiagnosed or underreported (Renukaradhya et al., 2002). As per estimates, the true incidence may be 25 times higher than reported possibly due to under-diagnosis (Smits and Kadri, 2005).

In this study, seropositivity was noticed in 4.25%, 3.54%, 6.02% and 4.96% samples by RBPT, STAT, Indirect ELISA and IgG ELISAin human sera samples, respectively. STAT titres above 1:160 are considered diagnostic in presence of compatible clinical presentation (Mantur et al., 2008). It has been opined that, a titre of 1:320 may be more specific in endemic areas (Smits and Kadri, 2005). In earlier studies, seropositivity of 2.26% 2.26%, 19.69% was reported by RBPT, SAT and Indirect ELISA, respectively among high risk groups in Karnataka (Agasthya et al., 2012). Similarly, seroprevalence of 4.79%, 4.41% and 4.41% was reported among cases of PUO by RBPT, SAT and 2ME, respectively in Bijapur, Karnataka (Mangalgi et al., 2012). Also *Brucella* strains were isolated from 26.4% cases. Brucellosis was detected in 0.8 to 3.3% cases of pyrexia of unknown origin (Handa et al., 1998; Kadri et al., 2000).

In Bangladesh, the prevalence of brucellosis was ranged between 2.5- 18.6% based on occupations of people (Islam et al., 2013). The prevalence rates were 2.6-21.6%, 18.6%, 2.5% and 5.3-1.1% in livestock farmers, in milkers, in butchers and in veterinarians, respectively, who had a history of direct contact with animals or consumption of raw milk (Islam et al., 2013). In Pakistan, among occupationally exposed groups, the general seroprevalence was reported to be 6.9% (Ali et al., 2013).

In this study, variable results were observed using the serological tests. The anibodies against *Brucella* were spotted in a traveler who was travelling internationally to Mediterranean countries regularly. The antibodies were detected by RBPT, STAT and ELISA. Travel-associated morbidity has been linked to brucellosis (Memish and Balkhy, 2004). New foods, drinks and exotic food preparations are experienced by travelers which may lead to their exposure to pathogens including *Brucella.* IgM ELISA did not show positivity in any of the sample tested possibly due to lack of active infection or chronic nature of the disease. In an earlier study, specific antibodies were not detected by the IgM ELISA in 10 patients who showed positivity by conventional tests (Go´mez et al., 2008). Also, owing to its low sensitivity, commercial IgM ELISA has a limited value in diagnosing brucellosis (Go´mez et al., 2008). Relevant differences among the tests may be attributed to the differences in the antigens employed in the assays.

None of the risk factor was found to be significantly related to prevalence of brucellosis in human population of Goa. However, knowledge about brucellosis and age of the person were related with seropositivity. Handling of cases of abortion in animals and proximity to neighborhoods had been found to be major risk factors for human brucellosis in Tanzania (John et al., 2010). It had also been observed that brucellosis had a significant association with untreated milk consumption, slaughtered meat, direct contact with animals and individuals who had a nomadic lifestyle in Iran (Alavi et al., 2014) and in individuals in high-risk group in Bangladesh (Rahman et al., 2012). Exposure to animals at home, handling aborted animals, slaughtering/butchering, and processing raw milk were highly significantly associated with the risk for brucellosis (Earhart et al., 2009). In Turkey, the risk factors for occupational exposure to brucellosis among veterinarians were handling deliveries of

animals and injury during *Brucella* vaccine administration (Kutlu et al., 2014). Close contact with livestock, consumption of fetus and placenta were the major risk factors for human brucellosis in Ecuador (Ron-Román et al., 2014). Milking sheep and goats, consumption of raw feta cheese made from sheep and goat milk and consumption of milk of cows and boiled feta cheese were the risk factors for human brucellosis in Jordan (Abo-Shehada and Abu-Halaweh, 2013).

This study reported significantly higher titers among PUO cases and occupationally exposed individuals (Fig. 3.1). Awareness about asymptomatic infection is essential for early diagnosis of brucellosis and prevention of infection. The region covered for investigation forms non-traditional livestock rearing region, therefore, occurrence of brucellosis in humans is of great public health significance. Lack of awareness among clinicians, farmers, animal handlers about this disease is affecting the rate of diagnosis. Also there is need to create awareness about consumption of raw milk, disposal of aborted fetuses and isolation of infected animals within the region.

3.5.2 Seroprevalence and risk factors for animals

Brucellosis is a highly significant and emerging veterinary public health problem in India due to ever developing dairy sector. As per latest livestock census of India (Anon, 2012), there was an increase in the population of cows by 6.52% over the previous census (2007) with a population of 122.9 million cows. Similarly, increase in the buffalo population was 7.99% over the previous census with the population of female buffaloes as 92.5 million. Bovine brucellosis is prevalent among most of the states in India which in turn is responsible for significant economic losses. But attempts to control the infection are less successful because strategies used

worldwide are not applicable in Indian scenario. Accurate prevalence estimates are essential to design control programs.

In this study, a total of 27.60%, 29.85% and 34.64% animals were detected as seropositive by RBPT, STAT and indirect ELISA, respectively. Earlier study in Goa (Barbuddhe et al., 2004) reported seropositivity of 37.38%, 36.45% and 40.78% among cattle by RBPT, STAT and AB-ELISA, respectively. Many reports from other states are available supporting this study. Recently Chand and Chhabra (2013) observed 22.34% and 34.15% individual animal seroprevalence by RBPT and Indirect ELISA in Haryana and Punjab, respectively. Jagapur et al. (2013) found 31.74% animals to be positive for brucellosis, which included 27.21% cattle and 36.34% buffaloes among the 3 states of India. Similarly, Trangadia et al. (2010) observed 22.18% and 13.78% seropositivity among bovines by ELISA and RBPT, respectively. Earlier study in Punjab reported seroprevalence of 12.09% among bovines by AB-ELISA (Dhand et al. 2005). Aulakh et al. (2008) detected seroprevalence of 20.67% in cattle and 16.41% in buffaloes by milk based ELISA.

Previous studies across the world showed variable seroprevalence rates. Sanogo et al. (2012) observed 10.3% seropositivity among cattle in Ivory Coast. Kashiwazaki et al. (2012) reported 21.5% prevalence in 3 districts of west Uganda and 3.4% in 2 districts of east Uganda by RBPT and indirect ELISA. Prevalence rates of 1.4% was reported in cattle of southeast Ethiopia (Gumi et al., 2013), 9.6% in northern plateau of North central Nigeria (Maurice et al. (2013) and 1.3% in Niger (Boukary et al., 2013).

In India, risk factors such as rapid movement of livestock for trade purpose, improper farm hygiene, lack of awareness about brucellosis has been found to be related to seropositivity among animals. In this study, lack of awareness about

brucellosis (OR=8.739, P=0.138) and inadequate floor space (OR=0.278, P=0.128) were significant risk factors for high prevalence brucellosis (Table 3.4). Seropositivity was observed on the farms having inadequate floor space because overcrowding facilitated the transmission of disease. Most of the farm owners were not aware of brucellosis and its zoonotic potential. Improper management of animal housing was observed in almost every farm. Risk behaviors involving overcrowding of farms, introduction of new animals without testing, improper disposal of aborted materials were observed. In Goa, trading of animals from neighboring states is promoted under government schemes. Available reports indicated that seroprevalence was very high among animals as well as humans in these regions of country. Unscreened animals were introduced to herd which was a potential threat to a healthy herd because the animal might carry various infectious organisms. Screening of animal herds for brucellosis prior to purchase is not a usual practice in Goa, mostly due to economic reasons. The high prevalence of brucellosis in cattle is potential threat to human health.

Chand and Chhabra (2013) observed that the most crucial risk factor which facilitated intra-herd spreading of brucellosis was abortion or delivery of an infected animal on the farm in Punjab, India. Boukary et al. (2013) reported that age of animal was significantly associated with seropositivity for brucellosis among cattle (P=0.05) in Niger. Age, sex, location, and herd size played a role in the epidemiology of brucellosis among cattle herds in Jigawa state, northwestern Nigeria (Mohammed et al., 2011) and large herd size having a history of abortions were found to be the risk factors for brucellosis among bovines in Uganda (Makita et al., 2011). Seropositivity for brucellosis was associated with a history of abortions in a herd, age of animals and herd size in Ivory Coast (Sanogo et al., 2012). Smaller herds with one to two cattle

were less seropositve as compared to large herds with more than eight cattle in Tajikistan (Lindahl et al., 2014) and the number of calvings per cow was associated with seropositivity.

The study indicated the high prevalence of brucellosis among cattle in different farms (Fig. 3.2). Varius risk factors played an important role for transmission and seropositivity among animals. Calfhood vaccination, culling of infected animals, regular screening of herds for brucellosis, maintenance of proper hygiene at dairy farms, proper disposal of aborted calves and awareness about public health impact of brucellosis may help in control and prevention of brucellosis.

Chapter 4
Isolation and identification of *Brucella* from clinical samples and their characterization by biochemical and molecular methods

Introduction

Brucellosis can be diagnosed accurately by isolation of the pathogen from clinical samples. In case of human samples, blood culture is considered as gold standard for diagnostic confirmation. *Brucella* organisms can also be isolated from pus, cerebrospinal, pleural joint, or ascitic fluid, and placental tissues (Etemadi et al., 1984; Doern, 2000). In general, blood culture for *Brucella* is performed by conventional Castaneda method where the blood specimen is directly inoculated in the liquid phase of the Castaneda medium and bottle is incubated in upright position. For subculture one does not have to open bottle instead the bottle is tilted so that liquid broth flows over the solid slant. It is again incubated in upright position. In acute cases colonies appear on slant.

Although the results by this method are satisfactory in acute untreated cases, the incubation time required is very long. The isolation rate is markedly reduced in treated cases or sub-acute/chronic brucellosis patients (Mantur and Mangalgi, 2004). Sensitivity of this method is 30-50% depending on *Brucella* species isolated. This method does not require frequent subculture on solid medium, hence, avoids chances of contamination. But this method is time consuming compared to automated systems and lysis centrifugation methods. Currently clot culture has gained attention as good option for isolation because isolation can be done from the same sample used for serology which avoids difficulties in obtaining repeat sample for culture.

In the last decade, automated blood culture systems have been introduced into clinical practice. These systems are based on continuous monitoring and colorimetric detection of CO_2 production by growing organisms (Maleknejad et al., 2007). Detection of *B. melitensis* was possible within the 7-day routine incubation period employing this technology. BACTEC 9240 and 9050 automated systems have proved

to be efficient for isolation of *Brucella* (Ruiz et al., 1997; Ozturk et al., 2002; Işeri et al., 2006).

Various selective culture media containing antibiotic supplements help to enrich growth of *Brucella* from clinical specimens. The presence of infection in animals could be confirmed by isolation the organism from aborted fetuses, vaginal secretions, blood, hygroma fluid and milk of infected animals (Godfroid et al., 2010; Alton et al., 1975). Vaginal swab taken immediately after abortion is the ideal source of *Brucella*. Also, fetal membrane, stomach contents and tissues are ideal materials of isolation. The infected animals secrete organisms in milk, hence, it can also be used for isolation. Selective enrichment is usually performed for body fluid samples. Aborted material is usually a rich source of bacteria thus can be directly inoculated on selective agar for isolation.

Brucella can be identified on basis of biochemical reactions. The organism is positive for catalase, oxidase, urease tests and H_2S production. Some strains show rapid urease production. *Brucella* is non-hemolytic bacterium, produces tiny cream coloured colonies on blood agar. The organism is Gram negative and shows tiny cocobacilli type morphology. The cells are arranged singly or in small groups. In MZN staining, the organism shows partial acid fast morphology. Genus specific PCR is more accurate and reliable method for identification of *Brucella*. Several targets have been evaluated for their sensitivity and specificity. The methods based on detection of specific sequences of 16S-23S genes, the *IS*711 insertion sequence and the *bcsp*31 gene encoding a 31-kDa protein have been validated (Ouahrani-Bettache et al., 1996; Baddour and Alkhalifa, 2008).

4.1 Sample Collection

4.1.1 Human

For culture of brucellae, blood samples (8 ml) were collected from 10 serologically positive patients (Table 3.1) aseptically. The samples were processed for blood culture within 24 h of collection.

4.1.2 Animals

4.1.2.1 Vaginal Swabs

Using sterile cotton swabs (Himedia, Labs) deep vaginal swabs were collected (Fig. 4.1). The swabs were immediately placed in collection tube containing Amies transport medium without charcoal (Himedia). The samples were transported in chilled conditions to the laboratory for further processing.

4.1.2.2 Vaginal discharge

After cleaning the vulvar region, the discharge (cervico-vaginal-mucus) was collected using the sterile swab (Hi-culture collection device, Himedia labs). The thick vaginal mucus was suspended in tube containing normal saline. The samples were transported under chilled conditions to the laboratory for further processing.

4.1.2.3 Venous blood

Blood samples (10 ml) were collected by jugular puncture of serologically positive animals. The samples were collected in plain sterile tubes using sterile 18 gauze needle. The blood was transported to the laboratory under chilled conditions for further processing.

4.1.2.4 Milk

The udder and teats were washed with disinfectant and wiped with clean cloth.

First few streams were discarded and 10 ml milk from each teat was collected in sterile 50 ml tubes.

4.1.2.5 Placental tissues

Part of placental tissues was excised by sterile scalpel and placed in sterile tube containing normal saline (Fig. 4.2). The samples were transported to laboratory under chilled conditions for further processing.

4.1.2.6 Tissues from aborted fetus

From the aborted fetus (at 7-8 months), the stomach was incised with scalpel and blade, and the contents of the fetal stomach were aspirated with the help of sterile disposable syringe (Fig. 4.3). Tissues from heart, spleen, and lung were excised by sterile scalpel and placed in sterile collection tubes containing normal saline. The samples were transported to laboratory under chilled conditions for further processing.

Table 4.1 Details of clinical samples collected from humans and animals

Sample type	Source	No. of samples
Venous blood	Human	10
Vaginal swabs	Cattle	88
Vaginal discharge	Cattle	12
Venous blood	Cattle	20
Milk	Cattle	72
Placental tissue	Cattle	10
Tissues from aborted fetus	Cattle	18

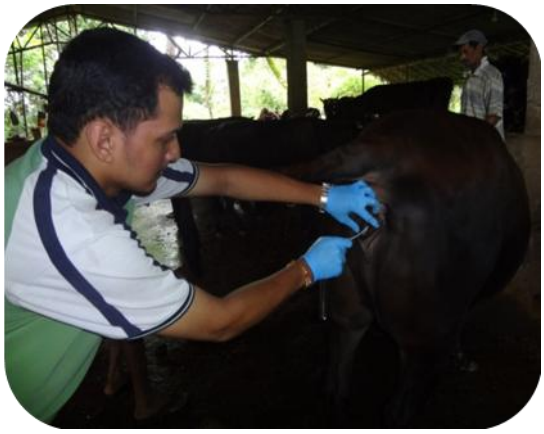


Fig 4.1 Collection of vaginal swab from cattle

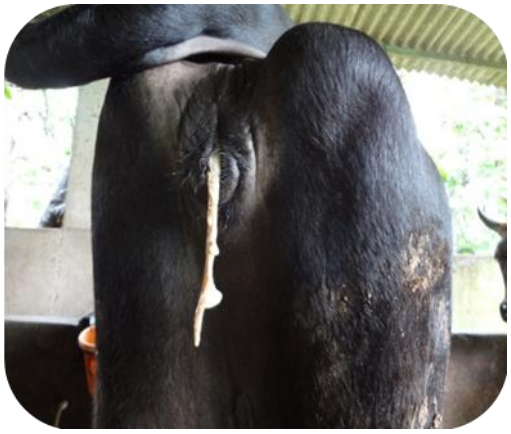
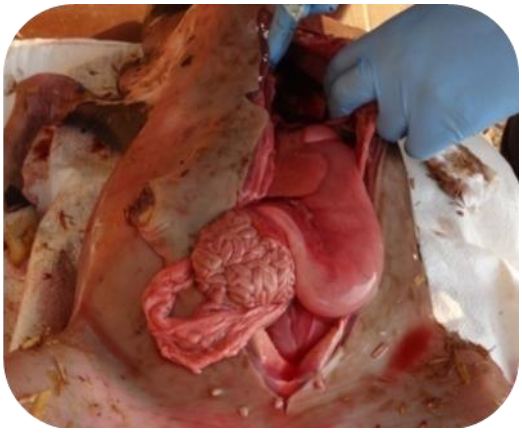


Fig 4.2 Collection of placental tissues from cattle showing retention of placenta



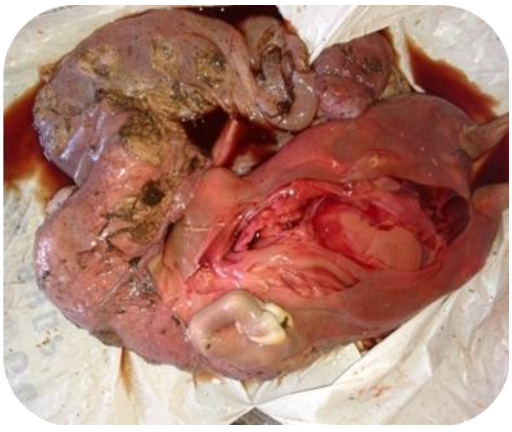


Fig 4.3 Collection of tissues from aborted fetus of cattle

4.2 Isolation of *Brucella*

4.2.1 Isolation from human samples by blood culture

Venous blood (8 ml) collected from patients was directly inoculated into 30 ml bottles of BD BACTEC^{TM} Plus Aerobic F/ medium (BD Biosciences, cat no. 442192).The bottles were incubated in the incubator rocker compartment of the BACTEC 9050 instrument upto 7 days. Sheep blood agar (5%) and *Brucella* agar with selective supplements (HiMedia Labs, Mumbai, India) were used for streaking the bacterial growth, if any, observed in the culture bottle and incubated at 37^0C. The plates were observed daily up to 8 days for evidence of bacterial growth.

4.2.2 Isolation from clinical samples collected from cattle

Isolation of the organisms was attempted as described by Alton et al. (1975). Placental tissues and tissues of aborted fetus were trimmed with sterile scissor and unwanted fat was removed. The tissues were directly placed on surface of *Brucella* agar containing selective supplement (Himedia Labs, Mumbai) and also placed overnight in *Brucella* selective broth for enrichment. The enriched broth was streaked on *Brucella* agar with selective supplements. The plates were incubated in 5% CO_2 atmosphere at 37^0C till growth appeared.

Vaginal discharge and stomach contents of aborted fetus were directly spread on surface of *Brucella* agar containing selective supplement (Himedia Labs, Mumbai). After centrifugation of milk for 5 min at 8000 rpm the upper cream and bottom deposit layer were spread separately on surface of *Brucella* agar containing selective supplement (Himedia Labs, Mumbai). The plates were incubated in 5% CO_2 atmosphere at 37^0C till growth appears.

Castaneda biphasic blood culture technique was used for isolation of *Brucella* from cattle blood (Castaneda, 1947). The biphasic apparatus consisted of *Brucella* agar and *Brucella* broth in solid and liquid phase respectively. Venous blood (5 ml) from cattle was aseptically inoculated to liquid phase of biphasic culture apparatus. Culture bottles were incubated in 5% CO_2 atmosphere at 37^0C for 7 days. The bottles were tilted after every 8 hr.

Vaginal swabs were inoculated in 5 ml of *Brucella* broth containing selective supplement for enrichment. Tubes were incubated at 37^0C till bacterial growth appeared. After appearance of bacterial growth, it was streaked on *Brucella* agar plates containing selective supplements and incubated at 37^0C till bacterial growth appeared.

Small circular glistening colonies with cream to honey colour appearance, entire margin, raised elevation and smooth consistency were regarded as presumptive *Brucella* isolate and subjected to identification.

4.3 Identification

4.3.1 Biochemical

Methods described by Alton et al. (1975) were used for biochemical identification. Gram staining, Modified Ziehl-Nielsen staining, hemolysis on 5% sheep blood agar, catalase, oxidase, urease, growth on MacConkey agar, nitrate reduction, methyl red, and Vogues Proskauer test were performed.

Gram negative isolates showing tiny coccobacilli type appearance and tiny, partially acid fast cocobacilli type appaearance in modified Ziehl Nielsen staining were regarded as belonging to genus *Brucella*. Non-hemolytic isolates with absence

of growth on MacConkey agar which showed positivity for catalase, oxidase, urease and nitrate reduction tests were identified as *Brucella* spp.

4.3.3 Identification by genus specific PCR

Phenol: chloroform method was used for extraction of genomic DNA from the isolates. The extracted DNA was quantified and checked for purity using Nano-Drop 1000 spectrophotometer (Thermo Scientific, USA). The PCR was performed for the detection of the *bcsp31* and *IS711* genes (Baily et al., 1992; Henault et al., 2000). The primers were synthesized from Sigma Aldrich. *B. abortus* S19 DNA and DNA from *E. coli* ATCC 8739 were used as positive and negative controls, respectively The reaction (25 µl) consisting of ready mix buffer with $MgCl_2$ (12.5 µl; Sigma Aldrich), 0.5 µl each forward and reverse primers (10 pmole), 1.5 µl of template DNA and 10 µl of nuclease free water were performed.

Table 4.2 Details of primers used for genus specific PCR.

Genes	Primer sequence	Amplicon	Ref.
bcsp31	F: TGGCTCGGTTGCCAATATCAA R :CGCGCTTGCCTTTCAGGTCTG	224 bp	Baily et al., 1992
IS711	F :CTGGCTGATACGCCGGACTTTGAA R :GGAACGTGTTGGATTGACCTTGAT	350 bp	Henault et al., 2000

The reaction was performed using thermalcycler (Eppendorf, Germany) with a preheated lid. The cycling condition for the *bcsp31* gene was initial denaturation at 95^0C for 3 min. This was followed by 35 cycles at 95^0C for 45 s of denaturation, annealing at 60^0C for 45 s and extension at 72^0C for 2 min with final extension at 72^0C for 10 min. The cycling condtions for the *IS711* gene were same as that of the

bcsp31 gene except the primer annealing at 55^0C. The PCR products were were electrophoresed using 1.5% agarose containing ethidium bromide and visualized under UV illumination (AlphaImager, USA). Isolates showing amplicons of both the *bcsp31* and *IS711* genes were confirmed as *Brucella* spp.

4.4 Results

4.4.1 Isolation

Table 4.3 Results of isolation from clinical samples

Sample	Source	No. of samples tested	No of isolates
Venous blood	Human	10	1
Venous blood	Cattle	20	1
Vaginal swabs	Cattle	88	16
Vaginal discharge	Cattle	12	3
Milk	Cattle	72	0
Placental tissue	Cattle	10	4
Tissues of aborted fetus	Cattle	18	4
Total		230	29

Twenty nine strains were isolated from clinical samples collected from bovines and humans (Table 4.3). Of these, 28 isolates were recovered from cattle vaginal swabs (n=16), vaginal discharge (n=3), placental tissues (n=4), venous blood from cattle (n=1) and tissues of aborted fetus (n=4) using selective media. One isolate was recovered from blood of a PUO patient by automated blood culture. Of the total isolates recovered from cattle, 57.14% isolates were recovered from vaginal swabs, 14.28% from placental tissue, 14.28% from tissues of aborted fetus and 3.57% from venous blood of cattle.

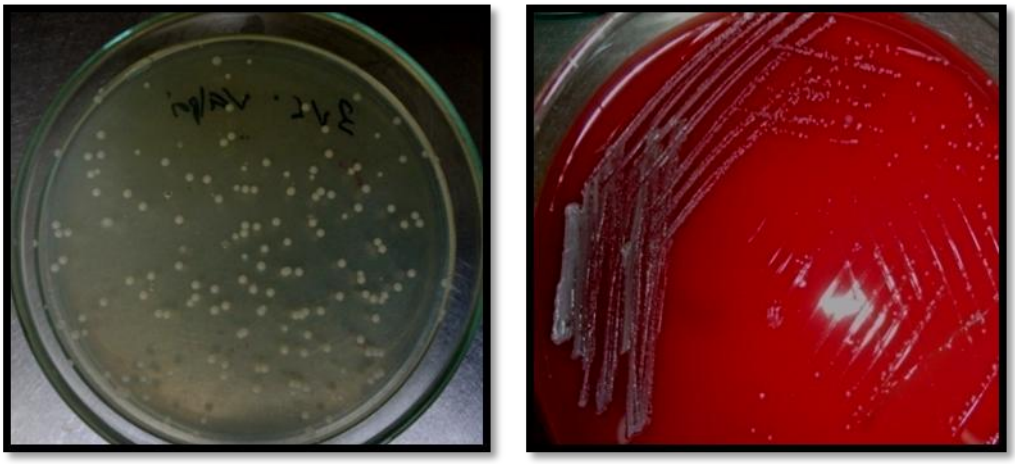


Fig 4.4.Colony morphology of *Brucella* spp.on *Brucella* agar with selective supplement (on left) and 5% sheep blood agar (on right)

4.4.2 Identification

4.4.2.1 Biochemical identification

All isolates (n=29) showed biochemical characteristic similar to *Brucella* spp. Some variation was observed in oxidase and nitrate reduction tests.

Table 4.4 Results of biochemical tests

Isolate	Catalase	Oxidase	Growth on MAC	Nitrate	Urease	MR	VP
1	+	+	-	+	+	-	-
2	+	+	-	+	+	-	-
3	+	+	-	+	+	-	-
4	+	+	-	+	+	-	-
5	+	-	-	+	+	-	-
6	+	+	-	+	+	-	-
7	+	+	-	+	+	-	-
8	+	+	-	-	+	-	-
9	+	+	-	+	+	-	-

10	+	+	-	+	+	-	-
11	+	+	-	+	+	-	-
12	+	+	-	+	+	-	-
13	+	-	-	+	+	-	-
14	+	+	-	+	+	-	-
15	+	+	-	-	+	-	-
16	+	+	-	+	+	-	-
17	+	+	-	+	+	-	-
18	+	+	-	+	+	-	-
19	+	-	-	+	+	-	-
20	+	-	-	+	+	-	-
21	+	-	-	+	+	-	-
22	+	+	-	+	+	-	-
23	+	+	-	+	+	-	-
24	+	+	-	+	+	-	-
25	+	-	-	-	+	-	-
26	+	-	-	+	+	-	-
27	+	+	-	+	+	-	-
28	+	+	-	+	+	-	-
29	+	+	-	-	+	-	-

Keys: +: Positive, -: negative

4.4.2.2 Identification by genus specific PCR

All the isolates (n=29) were shown to amplify the *bcsp31* and *IS711* genes. These genes are accepted as universal genetic markers for identification of *Brucella* spp.

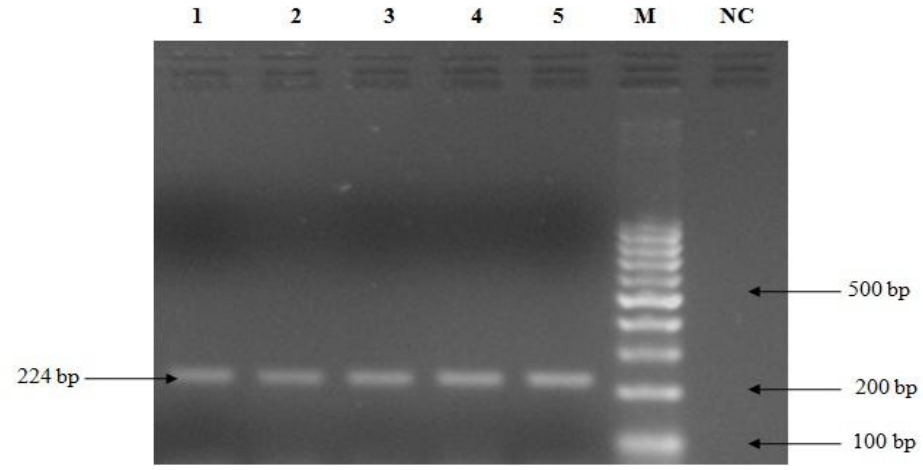


Fig 4.5 Amplification of *bcsp31* gene; Lanes 1-4: *Brucella* isolates, Lane 5: *Brucella abortus* strain 19 used as positive control, M: DNA ladder, NC: Negative control (*E. coli* ATCC 8739)

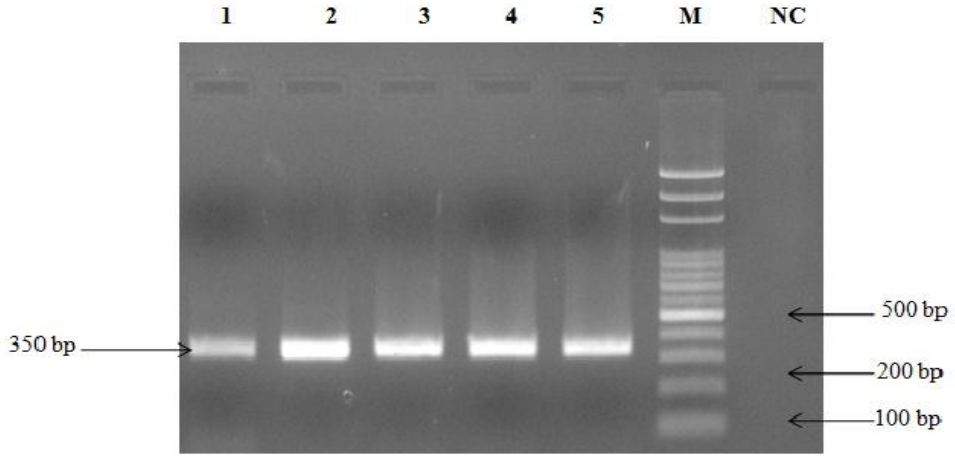


Fig 4.6 Amplification of *IS711* gene; Lanes 1-4: *Brucella* isolates, Lane 5: Positive control (*Brucella abortus* strain 19), M: DNA ladder, NC: Negative control (*E. coli* ATCC 8739)

4.5 Discussion

Isolation of brucellae by culturing blood and clinical specimens is essential for confirmation of the diagnosis. However, isolation of *Brucella* is not always successful due to fastidious nature of organism and presence of lower numbers of viable organisms in clinical specimens. Isolation is labour intensive and time consuming process. It requires level 3 biosafety conditions to avoid infection in laboratory workers.

Clinical samples were collected from animals for isolation of *Brucella*. The samples consisted of vaginal swabs (n=88), vaginal discharge (12), placenta (10), milk (n=72), venous blood (n=20) and tissues of aborted fetus (18). *Brucella* spp. was isolated from 28(12.72%) samples. A *Brucella* isolate was recovered from a patient having PUO after 72 h incubation in BACTEC blood culture system. The patient did not have significant IgG and IgM titres. The identifty of the pathogen was ascertained within 5 days of its culture. The period for isolation of the pathogen was significantly less than the classical biphasic culture technique used routinely.

Brucella agar with selective supplement proved to be useful media for isolation of *Brucella*. Enrichment culture of vaginal swabs in *Brucella* broth with selective supplement was also successful. The isolates were non-hemolytic on sheep blood agar (Fig. 4.4). Bacterial growth was not observed on MacConkey agar after 48 h. All isolates showed Gram negative, tiny cocobacilli appearance by staining. Isolates showed similarity with *Brucella* spp. in biochemical tests. Amplification of the *bcsp31* and *IS 711* genes was demonstrated by all isolates. Confirmation of the isolates as *Brucella* was done by biochemical and molecular methods.

Previous studies reported that *Brucella* spp. could be isolated from clinical samples of animals. Ali et al. (2014) recovered 30 isolates of *B. abortus* biovar 1 from milk (n=5), aborted fetuses (n=13), and vaginal swabs (n=12) in Pakistan. Most isolates were from cattle (56.7 %). The isolates were confirmed based on conventional biotyping methods and genus and species-specific PCR. In Sudan, Abdalla and Hamid (2012) reported *Brucella* spp. from 7.5% blood and milk samples, of which 22.4% samples were positive for the *IS711* gene. In another study, amplification of *Brucella*-DNA was observed from 55.76% bovine milk samples and *Brucella* were isolated from 46.15% samples of cow milk by direct culture method (Hamdy and Amin, 2002). Buyukcangaz and Sen (2007) obtained 8 *Brucella* isolates from aborted bovine fetuses in Turkey.

Isolation of *Brucella* spp. from clinical samples of infected animals is considered as a gold standard for diagnosis in animals. Abortion storms in cattle and buffalo were investigated and the 29 *Brucella abortus* isolates were obtained from morbid materials (Chand and Chhabra, 2013). Isolation of pathogen from the farms on which abortions had occurred indicated that disease was actively circulating among animals. Verma et al. (2000) isolated *B. abortus* biotype 3 from two of 110 samples from cows while, *B.melitensis* biotype 1 was recovered from three of 115 does and four of 163 samples collected from ewes. An outbreak of brucellosis was reported in an organized dairy farm in Himachal Pradesh (Chahota et al., 2003). Abortions, retained placenta and stillbirths were reported among 24 of the 290 animals in the farm. The *B. abortus* biotype-1 was isolated from placental tissues, discharges from uterus, vaginal swabs and stomach contents from foetus. Ghodsara et al. (2010) identified 5 out of 10 isolates of *B. abortus* by using *B. abortus* specific *bcsp31* and omp2a/2b primers. Trangadia et al. (2010) observed presence of *Brucella* spp. in 2 of

the 22 milk samples processed for isolation. Ghodsara and Roy (2012) obtained 10 *Brucella* isolates from cases of abortion and retention of placenta in cows, buffaloes, does and bitches in Gujarat state.

Isolation from human blood is critical for confirmation of disease. Escobar et al. (2013) isolated six *B. suis* strains from blood cultures of workers in a pig slaughterhouse. Hekmatimoghaddam et al. (2013) reported that blood culture was successful in 5% cases and 20.8% samples demonstrated amplification by PCR. *Brucella* spp. was isolated from a sputum sample of a patient suspected for TB using BACTEC system (Somily et al., 2013). Monitoring of blood cultures of 60 patients using BACTEC system (Ayaşlioğlu et al., 2004) revealed isolation of *Brucella* spp. from 26 of 31 patients by obtaining two blood cultures and in 17 of 29 patients attempting single blood cultures. Isolation was possible within 7 days of incubation in 84.1% cases, the earliest being the 3rd day in two cases.

A number of reports on isolation of brucellae from humans from India are available. When compared with the conventional Castaneda technique, the lysis centrifugation technique was more sensitive in both acute and chronic cases of human brucellosis (Mantur and Mangalgi, 2004). Isolation of *B. melitensis* was reported from 43 pediatric patients over a period of 13 years (Mantur et al., 2004). Comparison of lysis concentration (LC), clot culture and conventional Castaneda blood culture techniques for diagnosis of human brucellosis revealed better isolation of *Brucella* using blood, LC and clot culture techniques than the conventional technique (Mangalgi et al., 2014). Pawar et al. (2011) recovered *B. melitensis* from *Brucella* endocarditic case by blood culture and confirmed by PCR for the *omp2* gene.

In this study, *Brucella* isolates were recovered from vaginal swabs, placenta and tissues of aborted fetus. These are highly infectious materials and therefore

handling these materials needs care using protective barriers. Animal handlers should take proper precaution to avoid infection. As demonstrated in this study, *Brucella* isolates were recovered from a patient within 5 days by automated blood culture technique. Awareness among clinicians can significantly reduce the time required for diagnostic confirmation. Awareness among farmers, animal handlers, and consumers of unpasteurized food is required to control this infection.

Chapter 5
Determination of diversity among *Brucella* isolates by biotype identification, PFGE and virulence profiling

Introduction

Despite variations observed in host specificity, microbial phenotypes and pathogenicity, *Brucella* species show high levels of nucleotide similarity. Genus *Brucella* has 10 species infecting distinct hosts. The host range of the genus is continuously expanding. Recently, new strains were identified from terrestrial wildlife and marine mammals. *Brucella* species are subdivided into biotypes on basis of cultural, biochemical and serological properties of strains.

Currently, *B. abortus* has been biotyped in eight biovars (1-7 and 9), *B. melitensis* in three (1-3), and *B. suis* in five (1-5) (Osterman and Moriyon, 2006). The species and biotype identification of *Brucella* is based on carbon dioxide requirement, H_2S and urease productions, agglutination with A and M monospecific sera, growth in presence of thionin or basic fuchsin dyes, and phage typing (Al Dahouk et al., 2003; Alton et al., 1975). AMOS (*Abortus Melitensis Ovis Suis*) PCR is also useful for species and biotype identification (Bricker and Halling, 1994). This PCR uses a single reverse primer, targeting the *Brucella* specific insertion element *IS711*, and four different forward primers, each specific for a given species. Species are differentiated on the basis of different PCR fragment sizes (Scholz and Vergnaud, 2013).

Pulsed field gel electrophoresis (PFGE) is widely used in epidemiological studies and is a highly discriminative molecular typing technique. Using PFGE the variable migration of large DNA restriction fragments is detected in an electrical field of alternating polarity. By comparing the fingerprints of isolates, their clonality can be studied. The adoption of PFGE was facilitated by its use in international surveillance networks, such as PulseNet (CDC, USA) and standardization of protocols for important foodborne pathogens (*E. coli*, *Listeria*, *Campylobacter*). In spite of

availability of recent typing methods, PFGE is considered as the gold standard in a number of national and international surveillance programmes. PFGE using low-frequency cleavage enzymes has been suggested to give better discrimination between *Brucella* species (Jensen et al., 1999).

Brucellae are characteristic in several ways. The organism lacks classic virulence factors including capsules, secreted proteases, exo- and endotoxins, pili and fimbriae, and its atypical lipopolysaccharide pathogenicity (Crasta et al., 2008; DelVecchio et al., 2002). Virulence of brucellae mainly depends on their survival and replication in different cell types. The organisms control the maturation vacuoles to avoid innate immune responses and the replication in endoplasmic reticulum. Factors like LPS, Type IV secretion system, *Brucella* virulence factor A (bvfA), urease enzyme, cyclic β1,2-glucans have been found to be involved in virulence mechanisms of *Brucella* (Xavier et al., 2010).

Brucella virulence and intracellular replication is mediated by VirB protein forming the type IV secretion system (O'Callaghan et al., 1999; Foulongne et al., 2000; Comerci et al., 2001). In addition to this secretion system, the bvfA has also been suggested to be important for survival of *Brucella* in the host (Lavigne et al., 2005). These virulence factors can be detected by specific primers and probes and can be utilized for virulence based typing of *Brucella* isolates.

5.1 Biotype identification

5.1.1 Bacterial cultures

Standard bacterial cultures of *B. abortus* S19, *B. abortus* S99, *B. melitensis* Rev1, *B. suis* 2330 and *B. melitensis* biotype 1 were shared from Mumbai Veterinary College, Parel, Mumbai. *E. coli* ATCC 8739 was procured from IMTECH, Chandigarh, India.

5.1.2 Conventional biotyping

Conventional biotyping was performed as described (Alton et al., 1975). Growth in presence of CO_2, H_2S production, urease activity, growth inhibition on thionin and basic fuchsin containing media at 10 µg/ml (1:25000) and 20µg/ml (1:50000) concentrations were determined.

5.1.2.1 CO_2 requirement

The isolates were inoculated on agar slants in duplicates immediately after isolation. One slant was incubated aerobically and other at 5% CO2 atmosphere at 37^0C for 2-3 days. Growth on slant was monitored daily. If number of colonies on slant incubated in CO_2 atmosphere was greater that incubated aerobically, it was concluded that the culture required CO_2 for growth.

5.1.2.2 H_2S production

The cultures were inoculated in BHI broth tubes and incubated at 37^0C for 24 h. H_2S production was detected by placing the moist lead acetate strip (Hi media) on the neck of culture tube. If the culture is producing H_2S, the strip turns black.

5.1.2.3 Urease activity

Urease activity of the isolates was tested by inoculating isolates on Christensen's urea agar (Himedia Labs) slants. The slants were incubated at 37^0C for 18h. Change in colour of slant from cream to light pink was recorded as positive reaction.

5.1.2.4 Growth in presence of dyes

Stock solution (1%) of thionin and basic fuchsin (Himedia Labs) was placed in boiling water bath for 1 h after dissolving in sterile distilled water. Dyes were added to the molten trypticase soy agar (Himedia) in 10 µg/ml (1:25000) and 20µg/ml (1:50000) concentrations. The dyes were evenly mixed with the culture media and media was poured in plates. A loopful of recently grown standard cultures and field isolates were suspended in 1 ml of normal saline. A single streak across each dye plate and control plate containing plain medium was made using a sterile cotton swab after immersing in the suspension and incubated at 37^0C for 48 h. A total of 6 cultures were tested on a single plate. . Presence of growth on each dye plate with different concentrations was examined.

5.1.3 AMOS PCR

AMOS PCR was performed for identification of species and selected biotypes among genus *Brucella* (Bricker and Halling, 1994). The primers (Table 5.1) were synthesized from from Sigma Aldrich Chemicals. The PCR mixture (25 µl) comprised of ready mix with $MgCl_2$ (12.5 µl; Sigma Aldrich, USA), 0.5 µl of each of 4 primers for *B. abortus*, *B. melitensis*, *B. ovis* and *B. suis* (10 pmole/µl), 2 µl of *IS711* reverse primer (10 pmole/ µl), and 6.5 µl of sterile distilled water. To this mixture 2 µl of template DNA was added.

Table 5.1 Primers used in typing of *Brucella* by the AMOS PCR.

Primer	Sequence	Amplicon size (bp)
B. abortus F	GAC GAA CGG AAT TTT TCC AAT CCC	498
B. melitensis F	AAA TCG CGT CCT TGC TGG TCT GA	731
B. ovis F	CGG GTT CTG GCA CCA TCG TCG	976
B. suis F	GCG CGG TTT TCT GAA GGT TCA GG	285
IS711 R	TGC CGA TCA CTT AAG GGC CTT CAT	-

The PCR was carried out using thermalcycler (Eppendorf, Germany). The cycling conditions included initial denaturation at 95^0C for 2 min followed by 35 cycles of denaturation at 95^0C for 75 s, 55.5^0C for 2 min annealing and extension at 72^0C for 2 min. Final extension was performed at 72^0C for 5 min. The PCR amplicons were electrophoresed using 1.5% agarose gel containing ethidium bromide. The gel was visualized under UV illumination (AlphaImager, USA).

5.2 Pulsed field gel electrophoresis

The PFGE was done using protocol described by Brower et al. (2007) with suitable modifications. Briefly, the bacteria were grown for 48 h on trypticase soy agar (Himedia Labs, Mumbai). The growth was scraped in 400 µl cell suspension buffer (Appendix). The bacterial growth was measured and adjusted by Biophotometer (Eppendorf). A total of 200 µl of this adjusted cell suspension was mixed with 20µl of a 20mg/ml proteinase K solution (Sigma, St. Louis, MO). The cell suspension and 1% melted agarose in TE buffer (PFGE grade agarose, Biorad) maintained at 55°C were mixed by gentle pipetting. The part of mixture was dispensed immediately into wells of plug molds and kept for cooling for 10 to 15 min. The plugs were kept in lysis buffer consisted of 50 mM tris, 50 mM EDTA, 1% lauryl sarcosine, 0.1 mg/ml proteinase K, pH 8.0 at 55^0 C for 3 h in an orbital incubator

shaker. After lysis, the plugs were removed from the lysis buffer and washed in 15 ml pre-warmed (55^0C) sterile distilled water and then thrice with 15 ml of pre- warmed sterile TE buffer (10mM tris, 1 mM EDTA, pH 8.0) at 55^0C in an orbital incubator shaker for 15 min. The plugs were stored in TE buffer at 4°C after the final wash until used for restriction digestion.

A total of 200 µl of diluted restriction buffer (Thermo Scientific, USA) was added to each 1.5 ml sterile micro centrifuge tube. Each plug was placed into a separate tube containing restriction buffer. Five µl of *Xba*I restriction enzyme (1500U) (Thermo Scientific, USA) was added to each tube and kept for restriction digestion at 37^0C for 3 h. The plugs were then loaded to 0.5% Agarose (Certified Megabase Agarose, Biorad) gel in 0.5x TBE (45 mM each of Tris and borate, 1 mM EDTA) buffer and loaded on a CHEF-DR II apparatus (Bio-Rad, USA). The electrophoresis conditions used were: running time, 18.5 h; initial switch time, 1.0 s; final switch time, 12.0 s; ramp v/cm, 6. The gel was stained with ethidium bromide, and visualized under UV illumination (AlphaImager, USA) after destaining in distilled water. A lambda PFGE marker (New England Biolabs) was included in each of the run. The resultant PFGE patterns were analyzed using the Phoretix 1D pro software (Total Lab, UK). Pulsotypes of other *B. abortus* reference strains (n=1) and field strains (n=14) isolated from different locations in India, *B. melitensis* field strains (n=46), strains of *B. ovis* and *B. canis* were included for comparison. The pattern was clustured using the unweighted-pair group algorithm and the dice correlation coefficient.

5.3 Virulence profiling

The *virB* gene encoding for membrane bound type IV secretion system and the *bvfA* gene encoding *Brucella* virulence factor A were detected by PCR. The primers (Table 5.2) were synthesized from Sigma Aldrich, USA. The reaction mixture (25 µl) for the *virB* and *bvfA* genes included PCR ready mix Taq buffer with $MgCl_2$ (12.5 µl; Sigma Aldrich, USA), 1 µl of each of forward and reverse primers (10 pmole/µl), 2 µl of template DNA and 8.5 µl of nuclease free water.

Table 5.2.Details of primers used for detection of *Brucella* virulence genes

Gene	Primer sequence	Amplicon	Reference
bvfA	F: ACCCTTCGTCGATGTGCTGA R: CCGCGCTGATTTCATCGCTG	1282 bp	Lavigne et al. 2005
virB	F: CGCTGATCTATAATTAAGGCTA R: TGCGACTGCCTCCTATCGTC	881 bp	O'Callaghan et al.1999

The PCR assay was performed using Master Cycler epigradient thermal cycler (Eppendorf, Germany). The cycling conditions included initial denaturation at 95^0C for 4 min. This was followed by 35 cycles of denaturation at 95^0C for 60 s, annealing at 65^0C for 60 s for the *bvf*A gene and 54^0C for 60 s for the *vir*B gene, extension at 72^0C for 2 min. Finally, the primer extension was done at 72^0C for 10 min. The PCR amplicons were analyzed by electrophoresis on 1.5% agarose gel containing ethidium bromide and visualized under gel documentation system (AlphaImager, USA).

5.4 Results

5.4.1 Conventional biotyping

All the isolates were subjected to conventional biotyping and were confirmed as *B. abortus* biotypes 1, 2, 3 and 4. A total of 11 isolates were belonging to biotype 1, 8 were biotype 2, 6 were biotype 3 and 4 were belonging to biotype 4 (Table 5.3). The isolates demonstrated variable CO_2 requirement. All isolates were producing H_2S and urease enzyme. A total of 6 isolate demonstrated growth on both dyes basic fuchisn and thionin (At 1:25000 and 1:50000 concentrations). A total of 8 isolates were sensitive for both the dyes.

Table 5.3 Dye sensitivity results

Thionin 1:25000	**Basic Fuchsin 1:25000**	**Thionin 1:50000**	**Basic Fuchsin 1:50000**	**No. of isolates**
-	+	-	+	11
-	-	-	-	8
+	+	+	+	6
-	+	-	+	4

Table 5.4 Results of conventional biotyping tests

Species	**Biotype**	**No. of isolates**
Brucella abortus	1	11
Brucella abortus	2	8
Brucella abortus	3	6
Brucella abortus	4	4

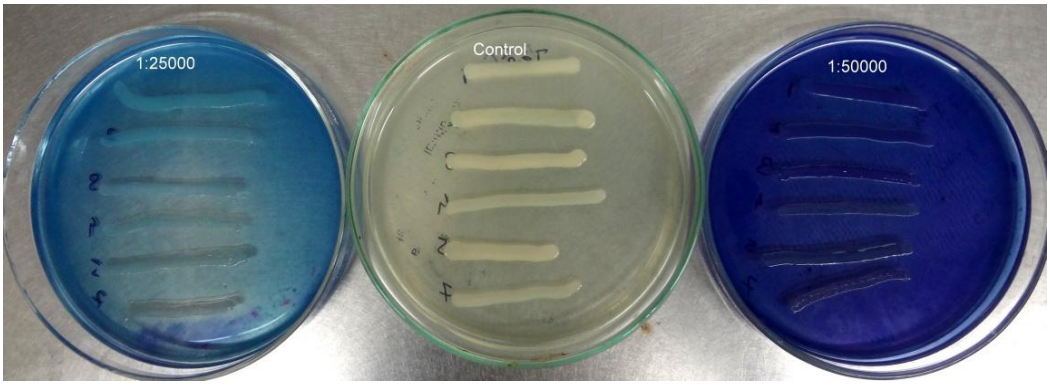


Fig. 5.1 Dye sensitivity test using thionin

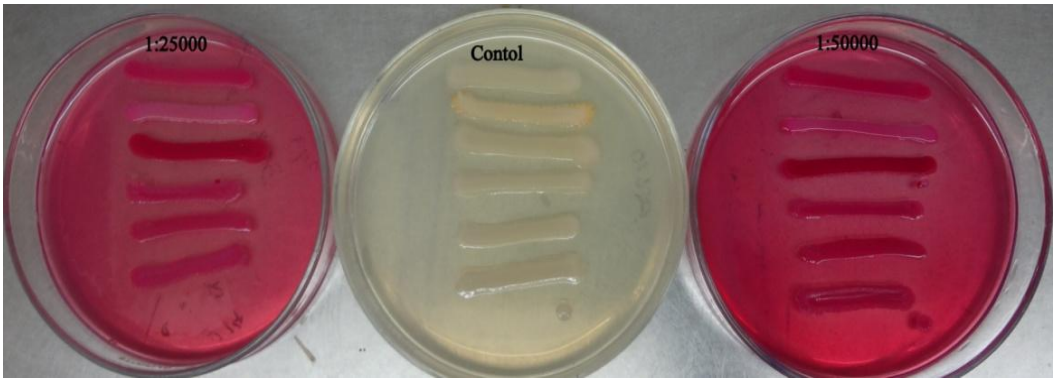


Fig 5.2 Dye sensitivity test using basic fuchsin

5.4.2 AMOS PCR

A total of 23 isolates demonstrated amplification of *B. abortus* specific primer (498 bp) in AMOS PCR . Of the total isolates, 6 isolates did not amplified any of the primer in AMOS PCR (Table 5.1).

Table 5.5 Results of AMOS PCR

Species	**Existing biovars**	**Biovars detected by AMOS PCR**	**No. of positive isolates**
B. abortus	1-6, 9	1,2, 4	23
B. melitensis	1-3	1,2, 3	0
B. ovis	1	1	0
B. suis	1-5	1	0

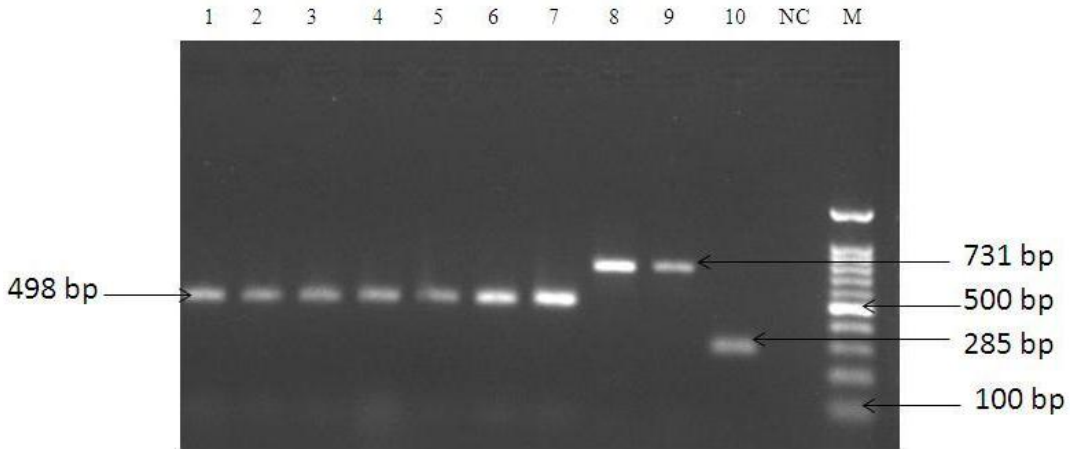


Fig 5.3 Amplification of *Brucella abortus* specific primer by *Brucella* isolates in AMOS PCR Lanes 1-5: *Brucella* isolates, Lane 6: *Brucella abortus* S19, Lane 7: *Brucella abortus* S99, Lane 8: *Brucella melitensis*, Lane 9: *Brucella melitensis* Rev1, Lane 10: *Brucella suis* 2330, Negative control: *E. coli* ATCC 8739

5.4.2 PFGE

A total of 29 isolates were subjected to PFGE for detection of diversiy (Fig. 5.4). Of these 20 isolates showed distinct PFGE profiles. Isolates formed 6 clusters at 60% similarity. A total of 18 pulsotypes were observed among the isolates (Fig. 5.5).

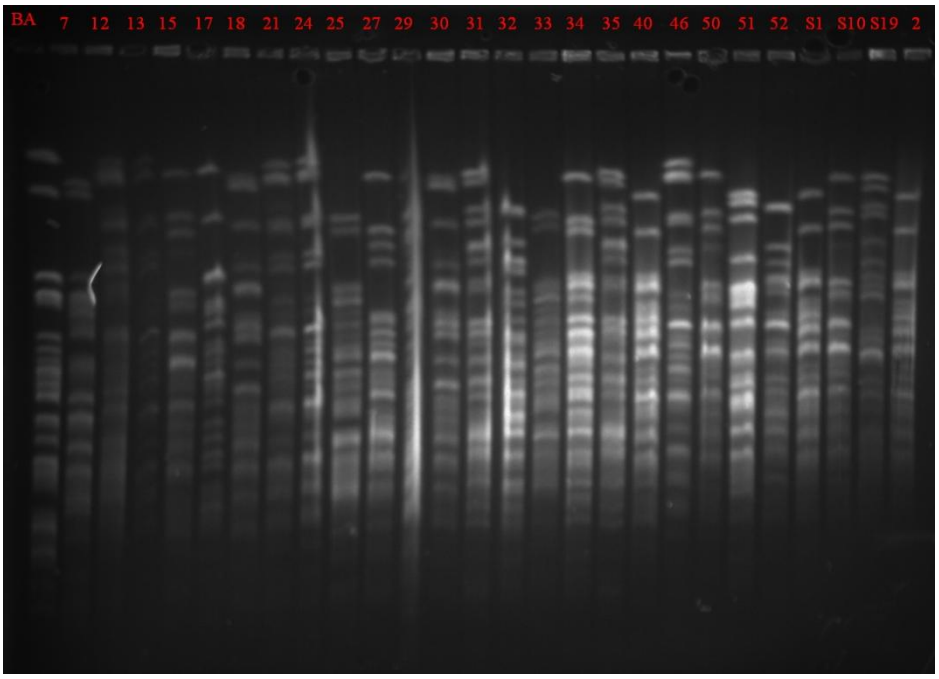


Fig 5.4 PFGE patterns of *Brucella* isolates obtained by *Xba*I restriction enzyme

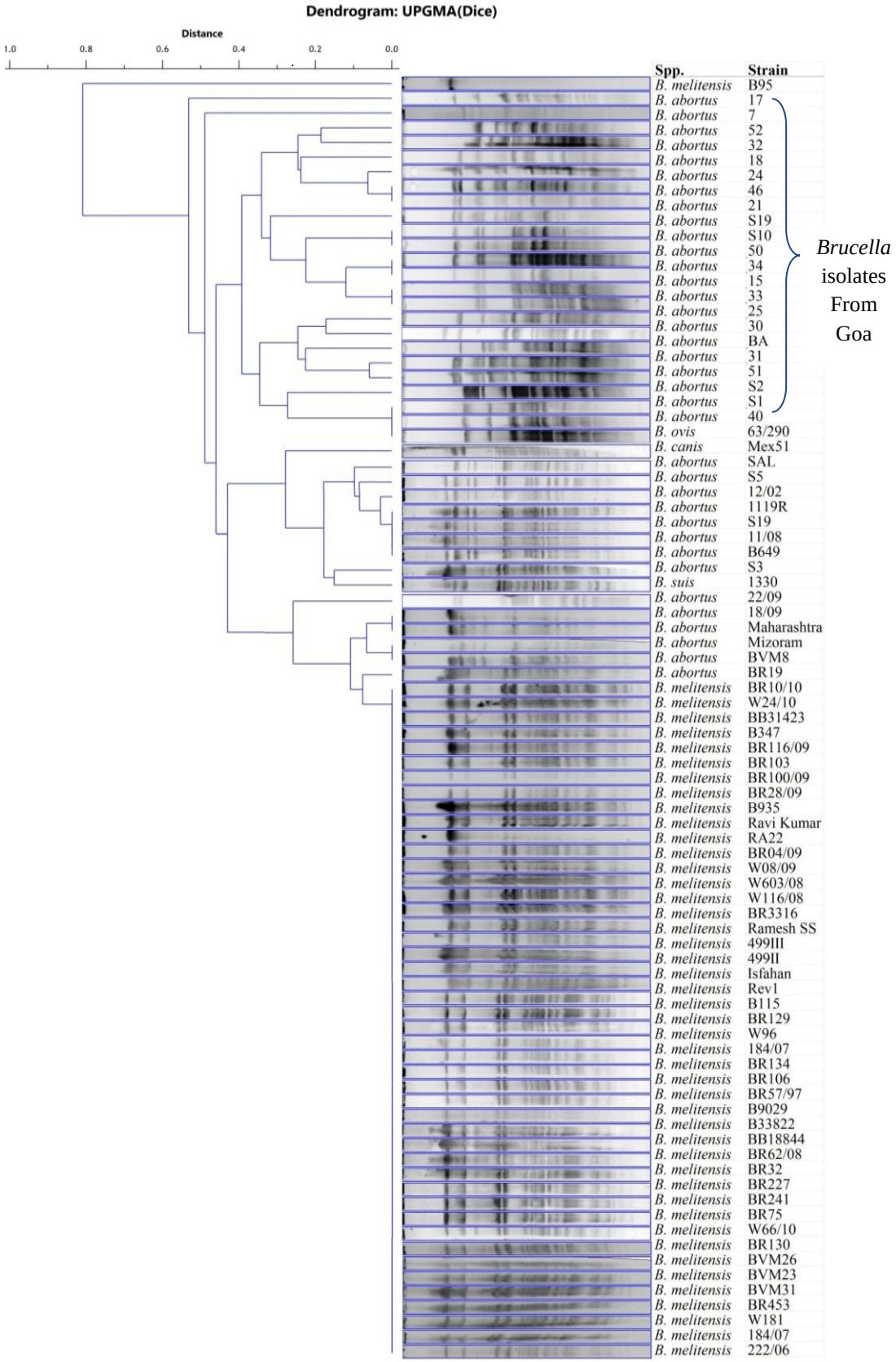


Fig 5.5 PFGE pattern of *Brucella* isolates by *Xba*I enzyme was compared with standard strains and field isolates from India. High similarity was observed within the isolates recovered from Goa. The pulsotypes of the isolates were different from other field isolates from India.

5.4.3 Virulence profiling

A total of 5 isolates demonstrated presence of the v*irB* (881 bp) and the *bvfA* (1282 bp) genes by PCR.

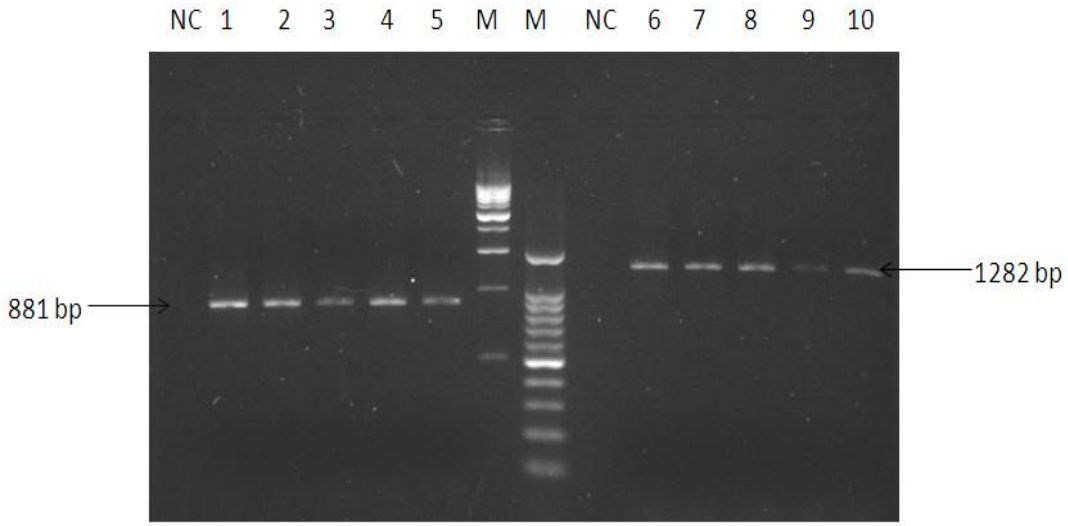


Fig 5.6 *Brucella* isolates showing amplification of virulence genes

Lanes 1-4: *virB* PCR products of *Brucella* isolates, Lane5: *Brucella abortus* S 19, Lanes 6-9: *bvfA* PCR products of *Brucella* isolates, Lane 10: *Brucella abortus* S19, M: 3 kb DNA ladder, M: 100 base pair DNA ladder

5.5 Discussion

Diversity within *Brucella* isolates was determined by biotyping, AMOS PCR and PFGE. Presence of virulence genes among the isolates was also checked by PCR. The *Brucella* strains isolated from human and bovine samples were biotyped using conventional and molecular subtyping methods. Five isolates demonstrated the presence of genes encoding type IV secretion system and *Brucella* virulence factor A.

Presence of different biotypes was checked by conventional biotyping tests and AMOS PCR. Conventional biotyping tests are useful for identification of biotypes but are found to be labour intensive and time consuming. Conventional biotyping indicated that isolates were *B. abortus* belonging to either biotype 1, 2, 3 or 4.

AMOS PCR was found to be convenient method for species and biotype identification. The assay confirmed that isolates were *B. abortus* belonging to either biotype 1, 2 or 4. Presence of biotype 3 was detected by conventional biotyping method only. Standard AMOS PCR can only detect biotype 1, 2 and 4. Thus, it can be opined that different biotypes of *B. abortus* were present among cattle farms of Goa. Presence of different biotypes indicated that different *Brucella* strains were being introduced unknowingly in this region.

Risk factors like unrestricted movement of animals for trade purpose, introduction of unscreened animals within herd were frequently observed in Goa region. Lack of awareness about brucellosis is responsible for spreading of this infection within healthy herds. Presence of diverse biotypes may arise due to mixing of herds. Thus it is recommended that animals should be screened prior to purchase to avoid infection of healthy animals.

This study indicated significant diversity within biotypes and pulsotypes among *B. abortus* isolates obtained from clinical samples in Goa. Previously, Ali et al. (2014) isolated 30 *Brucella* biovar 1 strains from cattle in Pakistan identified on basis of conventional biotyping methods. Langoni et al. (2000) reported prevalence of *B. abortus* in 30.61% milk samples by culture isolation in Brazil. Of these 2.04% isolates were belonging to biotype 1, 16.32% of biotype 2 and 12.25% were biotype 3. Out of the 397 isolates of *Brucella* recovered by culturing MRT positive milk samples, one, 356 and 40 isolates belonged to biotype 2, 3 and 9 (Zowghi et al. 1990). Priyantha (2011) reported 8 isolates belonging to biovar 3 of *B. abortus* among cattle herds in Srilanka. The presence of *B. abortus* was confirmed among cattle in Zimbabwe by AMOS PCR (Gomo et al., 2012). Mamisashvili et al. (2013) identified 33 bacterial isolates recovered from clinical samples of cattle in Georgia as *B.*

melitensis (12) and *B. abortus* (21) by AMOS PCR. In another study, 11 of the 14 *Brucella* isolates were confirmed as *B. abortus* biotypes 1 and 2 by conventional biotyping methods and AMOS PCR (Matope et al., 2009). Brucellosis outbreak in domestic elk was investigated by Her et al. (2010) in Korea. *Brucella* isolates (n=9) were recovered from slaughtered animals. AMOS PCR detected that all the 9 isolates were *B. abortus*.

Diversity within biotypes was also reported in India. Chahota et al. (2003) isolated *B. abortus* biotype-1 from placental tissues, discharges from uterus, vaginal swabs and stomach contents of foetuses in Himachal Pradesh. Verma et al. (2000) isolated *B. abortus* biotype 3 from 2 of 7 aborted cows while, *B.melitensis* biotype 1 was recovered from three of 115 does and four isolates *B. abortus* biotype 3 were recovered from 163 samples collected from ewes. Kaur et al. (2006) obtained 17 isolates of *B. abortus* from aborted materials of bovines, of which 11 belonged to biotype 1, 3 belonged to biotype 3 and 3 belonged to biotype 2. Nagalingam et al. (2012) reported 28 field isolates of *B. abortus* from Karnataka belonging to either biotype 1, 2 or 4 by AMOS PCR.

PFGE is a gold standard for molecular subtyping of pathogens. It is routinely used for outbreak investigations involving *E. coli*, *Salmonella*, *Shigella*, *Listeria* and many other pathogens of public health importance. It is mainly used to track the source of outbreak. Therefore, this method has a crucial importance in epidemiological investigations. A total of 20 *B. abortus* isolates and 2 reference strains (*B. abortus* S19, *B. abortus* S99) were analysed by PFGE using *Xba*I restriction enzyme. Distinct pulsotypes were observed by *Xba*I digestion (Fig 5.5). These pulsotypes were analysed by Phoretix 1D pro software. A total of 18 pulsotypes were obtained from 20 *B. abortus* isolates (Fig. 5.5). No predominant pulsotype was

observed. At 60% similarity, 6 clusters were formed. Significant diversity was observed (Simpson's index of diversity =0.83) among the *Brucella abortus* strains recovered from human and cattle clinical samples. The isolates demonstrated distinct diversity within biotypes. The pulsotypes were different from other field strains isolated from other parts of India.

A total of 6 clusters were observed among *B. abortus* isolates. Four clusters contained more than two isolates (major cluster). Two isolates were placed singularly in upper part of the dendrogram. Cluster 1 contained isolates S1, S2 and 40. Cluster 2 consisted isolates 51, 31, 30, 25 and *B. abortus* reference strain. Cluster 3 consisted *B. abortus* S19, S10, 50, 34, 15, 33. Cluster 4 consisted of isolate no. 52, 32, 18, 24, 46, and 21. Cluster 5 and 6 contained isolate no.7 and 17 respectively. Previously, Brower et al. (2007) compared different techniques involving PFGE, ribotyping and OMPA for strain differentiation of outbreak associated *B. canis* isolates from Wisconsin kennels in USA. All the *B. canis* isolates were similar genetically and were not able to be differentiated by ribotyping, PFGE and OMPA. Li et al. (2013) observed 6 PFGE clusters within 32 *B. suis* isolates collected from China by *Xba*I restriction digestion.

Use of various phenotypic and genetic tools plays imperative role in studying the diversity among *Brucella* spp. and epidemiology of the disease. Phenotypic methods though reliable, are labour intensive and sometimes provide ambiguous results. Molecular tools provide results in much shorter time. Homogenous nature of the genus *Brucella* has restricted the use of genetic diversity tools.

Summary and Conclusions

Brucellosis is a common zoonosis with worldwide distribution. The disease has been eradicated from many developed countries. But developing countries in Asia, Africa, and Mediterranean basin are facing significant economic losses due to this infection. Brucellosis among animals is overlooked due to lack of awareness. The disease is under diagnosed or confused with other diseases in humans due to common clinical symptoms. Lack of awareness about mode of transmission and risk factors for this disease among clinicians, farmers, policy makers and general public are the major impediments for prevention of this infection. As a result of complex epidemiology of the disease, it is still not known in many countries.

Brucellosis is endemic in India. The disease has been reported in cattle, buffaloes, sheep, goats, pigs, yaks, camels, mithun and other livestock. Human brucellosis has been detected among occupationally exposed groups involving veterinarians, animal handlers, butchers, meat inspectors as well as in cases of pyrexia of unknown origin (PUO). Many seroprevalence studies have been performed across all the states indicating variable prevalence of the disease. But distribution of biotypes and diversity within the *Brucella* strains is unknown due to poor recovery of viable organisms from clinical samples. Recently, improved culture techniques have been developed to isolate the organisms from different matrices. Selective culture media are facilitating the exclusion of contaminating micro flora. Blood culture is routinely practiced for recovery of pathogen from human samples. Automated blood culture methods are able to recover organism rapidly thus, reducing the diagnostic delay.

Goa is a smallest state in India bestowed by natural resources. Animal husbandry is most important allied business in agriculture and provides major source of income for farmers because vast majority of the human population is dependent on animal originated foods as source of protein. Livestock population of Goa includes

cattle, buffaloes, goat, pigs and poultry. Animal husbandry is facing losses due to lack of knowledge about farm hygiene, sanitation, proper housing conditions which are major risk factors for transmission various infections including brucellosis. Unscreened animals are unknowingly introduced within herds which may act as potential carriers of pathogens. Therefore, large numbers of people are at a greater risk of infection as they are regularly exposed to animals. Prevalence of brucellosis in Goa is unknown. Therefore, the study was focused on determination of epidemiological status of brucellosis among cattle and human population in Goa.

- Human blood samples (n=282) from cases of PUO (n=243) and individuals with occupational exposure (n=39) were collected from Goa Medical College and Hospital, and private hospitals from Goa. Blood samples (n=355) were collected from 12 different cattle farms in Goa. The farms were selected on basis of incidences of abortions reported to state veterinary officials. The samples were collected from aborted animals, animals with reproductive disorders as well as in contact healthy animals.

- The samples were tested by RBPT, STAT and indirect ELISA. Human samples were further tested by IgG and IgM ELISA. A total of 25(8.86%) human serum samples were positive to at least one of the test employed. Seropositivity was detected in 4.25%, 3.54%, 6.02% and 4.96%, human samples by RBPT, STAT, Indirect ELISA and IgG ELISA, respectively. No serum sample demonstrated presence of the IgM titers. Similarly, seropositivity was detected in 27.60% by RBPT, 29.85% by STAT, 34.64% by Indirect ELISA in cattle sera.

- A total of 220 clinical samples consisting of vaginal swabs (n=88), vaginal discharge (n=12), placenta (n=10), milk (n=72), venous blood (n=20) and tissues

of aborted fetus (n=18) were collected from cattle. Samples were processed for isolation of *Brucella* using selective culture media. A total of 28 isolates were recovered from cattle vaginal swabs (n=16), vaginal discharge (n=3), placental tissues (n=4), venous blood from cattle (n=1) and tissues of aborted fetus (n=4).

- Of the 10 blood samples from serologically positive PUO patients subjected for isolation, one *Brucella* strain was isolated using by BACTEC blood culture.

- Identification was performed by biochemical tests and genus specific PCR. All the *isolates w*ere identified as *Brucella* spp. using biochemical tests. The isolat*es (n*=29) d*emons*trated presence of the *bcsp31* and *IS711* genes which are important m*olecular* targets for identification of *Brucella* spp.

- Diversity within biotypes among *Brucella* isolates was investigated by conventional biotyping tests. AMOS PCR was performed for identification of species and selected biotypes within genus *Brucella*. Conventional tests indicated that isolates were *Brucella abortus* belonging to either biotypeas 1, 2, 3 and 4. A total of 11 isolates belonged to biotypes 1, 8 were biotype 2, 6 were biotype 3 and 4 belonged to biotype 4. A total of 23 isolates demonstrated amplification of *B. abortus* specific primer (498 bp) in AMOS PCR indicating either biotype 1, 2 or 4.

- Pulsed field gel electrophoresis using *XbaI* enzyme was performed to detect genetic diversity among *Brucella* isolates. A total of 29 isolates were subjected to PFGE for detection of diversiy. Of these, 20 isolates showed distinct PFGE profiles. Isolates formed 6 clusters at 60% similarity. A total of 18 pulsotypes were observed among the isolates. The isolates demonstrated distinct diversity

within biotypes. The pulsotypes were different from field strains isolated from other parts of India.

- *Brucella* isolates (17.24%) demonstrated the presence of the *virB* gene encoding type IV secretion system, and the *bvfA* gene encoding *Brucella* virulence factor A by PCR.
- Risk factors for brucellosis among cattle and human population in Goa were identified through field visits. A questionnaire providing epidemiological data was prepared. A survey for identification of risk factors was conducted. Lack of awareness about brucellosis (OR=8.739, P=0.138) and inadequate floor space (OR=0.278, P=0.128) were significant risk factors for high prevalence brucellosis among cattle. None of the risk factor demonstrated significant correlation for brucella infection among human population under study. However, knowledge about brucellosis and age of the person were found to be related with seropositivity.

The present study detected significant seroprevalence of brucellosis among human and animal population of Goa. Higher prevalence was detected in cattle than humans. *B. abortus* was isolated from clinical samples of cattle in different farms indicating that disease is circulating among the population. One isolate of *B. abortus* was recovered from blood sample of a patient. This showed that awareness among clinicians could reduce the time required for diagnosis. Isolates were possessing virulence genes and demonstrated diversity in biotypes and PFGE pattern. Therefore, this study concluded that diverse *Brucella* strains were circulating among human and animal populations of Goa.

Future Perspectives

Brucella is an intracellular pathogen which can persist in animal and human population resulting in severe morbidity and economic losses. The burden of brucellosis is unknown in most of the developing countries. The genus is expanding in form of host range and reservoirs. Therefore studying the disease epidemiology and phylogeny is particularly important. Present study revealed significant prevalence of brucellosis among dairy cattle and human population of Goa. *Brucella* isolates were recovered from clinical samples of cattle and human. Presence of *Brucella abortus* biotype 1, 2, 3 and 4 were confirmed by biochemical tests and PCR. However PFGE data indicated that diverse *Brucella* strains are circulating among human and animal population.

For further subtyping of the isolates MLST and MLVA need to be used. MLVA is a reliable discriminatory tool in trace-back analyses and outbreak studies. MLVA using 15, 16 or 21 loci need to be performed to identify diversity within isolates. MLVA genotype data will be useful for constructing *Brucella* epidemiology database. This data will help in future studies for identification of outbreak causing strain or to trace source of outbreak. MLST is widely used as tool for characterization and typing of different bacterial species. MLST based on housekeeping or virulence genes will help to define genetic relatedness of isolates. It will also help to identify unknown *Brucella* isolates and determine their phylogenetic relationship.

Pathogenesis of *Brucella* spp. is poorly understood. *Brucella* has a non classical way of infecting host. Very few virulence factors have been identified till date. Therefore it is customary to detect potential virulence factors which are responsible for intracellular survival of *Brucella*. Whole genome sequencing will be the best approach to identify virulence factors candidate.

India has a rich wildlife heritage. Today the nation is having significant mammal population (340 species) on land and in seas. As potential novel *Brucella* species have been reported in wildlife across the globe, it is essential to explore wildlife reservoirs for *Brucella* spp. in India.

Though the Goa region forms a non-traditional area for livestock rearing, the high prevalence of brucellosis among animals is of public health concern owing to its zoonotic nature. Different risk factors responsible for transmission and spreading of this disease have been identified during field surveys. Lack of awareness about brucellosis and inadequate floor space at farms were significant risk factors for brucellosis in bovines. A case control study is required for management of brucellosis in Goa. Screening of animal herds for brucellosis prior to purchase of animals needs to be emphasized. Calf- hood vaccination is ideal way of controlling brucellosis among herds. Correlation between calf-hood vaccination, test and isolation policy and seropositivity need to be established.

Bibliography

Abdalla, A., and Hamid, M. E., 2012. Comparison of conventional and non-conventional techniques for the diagnosis of bovine brucellosis in Sudan. Tropical Animal Health Production, 44(6), 1151-1155.

Abdollahi, A., Morteza, A., Khalilzadeh, O., Rasoulinejad, M., 2010. Brucellosis serology in HIV-infected patients. International Journal of Infectious Diseases, 14(10), e904-906.

Abernethy, D. A., Menzies, F. D., McCullough, S. J., McDowell, S. W., Burns, K. E., Watt, R., Gordon, A. W., Greiner, M., Pfeiffer, D. U., 2012. Field trial of six serological tests for bovine brucellosis. Veterinary Journal, 191(3), 364-370.

Abo-Shehada, M. N., and Abu-Halaweh, M., 2011. Seroprevalence of *Brucella* species among women with miscarriage in Jordan. East Mediterranean Health Journal, 17(11), 871-874.

Abo-Shehada, M. N., and Abu-Halaweh, M., 2013. Risk factors for human brucellosis in northern Jordan. East Mediterranean Health Journal, 19(2), 135-140.

Ackermann, M. R., Cheville, N. F., Deyoe, B. L., 1988. Bovine ileal dome lymphoepithelial cells: endocytosis and transport of *Brucella abortus* strain 19. Veterinary Pathology, 25(1), 28-35.

Adone, R., Ciuchini, F., Marianelli, C., Tarantino, M., Pistoia, C., Marcon, G., Petrucci, P., Francia, M., Riccardi, G., and Pasquali, P. 2005. Protective properties of rifampin-resistant rough mutants of *Brucella melitensis*. Infection and immunity, 73(7), 4198-4204.

Adugna, K. E., Agga, G. E., Zewde, G., 2013. Seroepidemiological survey of bovine brucellosis in cattle under a traditional production system in western Ethiopia. Revue Scientifique Et Technique Office International des Epizooties, 32(3), 765-773.

Agasthya, A. S., Isloor, S., Krishnamsetty, P., 2012. Seroprevalence study of human brucellosis by conventional tests and indigenous indirect enzyme-linked immunosorbent assay. The Scientific World Journal, 2012, 104239.

Agasthya, A. S., Isloor, S., Prabhudas, K., 2007. Brucellosis in high risk group individuals. Indian Journal of Medical Microbiology, 25(1), 28-31.

Ahmed, M. O., Elmeshri, S. E., Abuzweda, A. R., Blauo, M., Abouzeed, Y. M., Ibrahim, A. Salem, H., Alzwam, F., Abid, S., Elfahem, A., Elrais, A., 2010. Seroprevalence of brucellosis in animals and human populations in the western mountains region in Libya, December 2006–January 2008. Eurosurveillance, 15, 30, pii19625.

Ajay Kumar, V. J., Nanu, E., 2005. Sero-positivity of brucellosis in human beings. Indian Journal of Public Health, 49(1), 22-24.

Al Dahouk, S., Flèche, P. L., Nöckler, K., Jacques, I., Grayon, M., Scholz, H. C., Tomaso, H., Vergnaud, G., Neubauer, H., 2007. Evaluation of *Brucella* MLVA typing for human brucellosis. Journal of Microbiological Methods, 69(1), 137-145.

Al Dahouk, S., Sprague, L. D., Neubauer, H., 2013. New developments in the diagnostic procedures for zoonotic brucellosis in humans. Revue Scientifique Et Technique Office International des Epizooties, 32(1), 177-188.

Al Dahouk, S., Tomaso, H., Nöckler, K., Neubauer, H., Frangoulidis, D., 2003. Laboratory-based diagnosis of brucellosis--a review of the literature. Part II: serological tests for brucellosis. Clinical Laboratory, 49(11-12), 577-589.

Al Dahouk, S., Tomaso, H., Prenger-Berninghoff, E., Splettstoesser, W. D., Scholz, H. C., Neubauer, H. 2005. Identification of *Brucella* species and biotypes using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Critical Reviews in Microbiology, 31(4), 191-196.

Al-Ani, F.K., El-Qaderi, S., Hailat, N. Q., Razziq, R., Al-Darraji, A. M., 2004. Human and animal brucellosis in Jordan between 1996 and 1998: a study. Revue Scientifique et Technique Office International des Epizooties 23(3), 831-840.

Alavi, S. M., Mugahi, S., Nashibi, R., Gharkholu, S., 2014. Brucellosis risk factors in the southwestern province of Khuzestan, Iran. International Journal of Enteric Pathogens, 2(1), e15610.

Ali, S., Ali, Q., Melzer, F., Khan, I., Akhter, S., Neubauer, H., Jamal, S. M. 2014. Isolation and identification of bovine *Brucella* isolates from Pakistan by biochemical tests and PCR. Tropical Animal Health Production, 46(1), 73-78.

Ali, S., Ali, Q., Neubauer, H., Melzer, F., Elschner, M., Khan, I., Abatih, E. N., Ullah, N., Irfan, M., Akhter, S. 2013. Seroprevalence and risk factors associated with brucellosis as a professional hazard in Pakistan. Foodborne Pathogens and Diseases, 10(6), 500-505.

Al-Kharfy, T. M., 2001. Neonatal brucellosis and blood transfusion: case report and review of the literature. Annals of Tropical Pediatrics, 21(4), 349-352.

Allan, G. S., Chappel, R. J., Williamson, P., McNaught, D. J., 1976. A quantitative comparison of the sensitivity of serological test for bovine brucellosis to different antibody classes. Journal of Hygiene (London), 76(2), 287-298.

Allardet-Servent, A., Bourg, G., Ramuz, M., Pages, M., Bellis, M., Roizes, G., 1988. DNA polymorphism in strains of the genus *Brucella*. Journal of Bacteriology, 170(10), 4603-4607.

Al-Majali, A. M. and Shorman, M., 2009. Childhood brucellosis in Jordan: prevalence and analysis of risk factors. International Journal of Infectious Diseases, 13(2), 196-200.

Al-Majali, A. M., Talafha, A. Q., Ababneh, M. M., Ababneh, M. M., 2009. Seroprevalence and risk factors for bovine brucellosis in Jordan. Journal of Veterinary Science, 10(1), 61-65.

Al-Shaar, L., Chaaya, M., Ghosn, N., Mahfoud, Z., 2014. Brucellosis outbreak in Chouf district of Lebanon in 2009: a case-control study. East Mediterranean Health Journal, 20(4), 250-256.

Alton G., Jones L., Pietz D., 1975. Laboratory techniques in brucellosis .2nd edition. World Health Organization, Geneva, Switzerland.

Amin, K. M., Rahman, M. B., Rahman, M. S., Han, J. C., Park, J. H., Chae, J. S., 2005. Prevalence of *Brucella* antibodies in sera of cows in Bangladesh. Journal of Veterinary Science, 6(3), 223-226.

Anderson, T. D., Cheville, N. F., 1986. Ultrastructural morphometric analysis of *Brucella abortus*-infected trophoblasts in experimental placentitis. Bacterial replication occurs in rough endoplasmic reticulum. American Journal of Pathology, 124(2), 226-237.

Angel, M. O., Ristow, P., Ko, A. I., Di-Lorenzo, C. 2012. Serological trail of *Brucella* infection in an urban slum population in Brazil. Journal of Infection in Developing Countries, 6(9), 675-679.

Aniyappanavar, D., Prasad, S. R., Tanveer, K. M., Rao, S., 2013. *Brucella* infections in high-risk population and in patients hospitalized for fever: A serological study at Kolar, Karnataka. Annals of Tropical Medicine and Public Health, 6(5), 49-53.

Anka, M. S., Hassan, L., Adzhar, A., Khairani-Bejo, S., Mohamad, R. B., Zainal, M. A., 2013. Bovine brucellosis trends in Malaysia between 2000 and 2008. BMC Veterinary Research, 9(1), 230.

Anon, 2012. 19th Livestock Census, 2012. All India Report. Ministry of Agriculture Department of Animal Husbandry, Dairying and Fisheries Krishi Bhawan, New Delhi.

Appannanavar, S. B., Sharma, K., Verma, S., Sharma, M., 2012. Seroprevalence of Brucellosis: A 10-year experience at a tertiary care center in north India. Indian Journal of Pathology and Microbiology, 55(2), 271-272.

Arellano-Reynoso, B., Lapaque, N., Salcedo, S., Briones, G., Ciocchini, A. E., Ugalde, R., Moreno, E., Moriyón, I., Gorvel, J. P., 2005. Cyclic beta-1, 2-glucan is a *Brucella* virulence factor required for intracellular survival. Nature Immunology, 6(6), 618-625.

Ariza, J., Gudiol, F., Pallarés, R., Rufí, G., Fernández-Viladrich, P., 1985. Comparative trial of rifampin-doxycycline versus tetracycline-streptomycin in the therapy of human brucellosis. Antimicrobial Agents and Chemotherapy, 28(4), 548-551.

Asaad, A. M., Alqahtani, J. M., 2012. Serological and molecular diagnosis of human brucellosis in Najran, Southwestern Saudi Arabia. Journal of Infection and Public Health, 5(2), 189-194.

Asmare, K., Sibhat, B., Molla, W., Ayelet, G., Shiferaw, J., Martin, A. D., Skjerve, E., Godfroid, J., 2013. The status of bovine brucellosis in Ethiopia with special emphasis on exotic and cross bred cattle in dairy and breeding farms. Acta Tropica, 126(3), 186-192.

Atluri, V. L., Xavier, M. N., de Jong, M. F., den Hartigh, A. B., Tsolis, R. M., 2011. Interactions of the human pathogenic *Brucella* species with their hosts. Annual Reviews of Microbiology, 65, 523-541.

Aulakh, H. K., Patil, P. K., Sharma, S., Kumar, H., Mahajan, V., Sandhu, K. S., 2008. A study on the epidemiology of bovine brucellosis in Punjab (India) using milk-ELISA. Acta Veterinaria Brno, 77, 393-399.

Aworh, M. K., Okolocha, E., Kwaga, J., Fasina, F., Lazarus, D., Suleman, I., Poggensee, G., Nguku, P., Nsubuga, P. 2013. Human brucellosis: seroprevalence and associated exposure factors among abattoir workers in Abuja, Nigeria - 2011. The Pan African Medical Journal, 16, 103.

Ayaşlioğlu, E., Kiliç, D., Kaygusuz, S., Küçük, S., Ceken, S., Erol, O., Alpay, Y., 2004. The detection of *Brucella* spp by BACTEC 9050 blood culture system. Bulletin of Microbiology, 38(4), 415-419.

Baba, M. M., Sarkindared. S. E., Brisibe, F. 2001. Serological evidence of brucellosis among predisposed patients with pyrexia of unknown origin in the north eastern Nigeria. Central European Journal of Public Health, 9(3), 158-161.

Baddour M. M., 2012. Diagnosis of Brucellosis in Humans: a Review. Journal of Veterinary Advances, 2(4), 149-156.

Baddour, M. M. and Alkhalifa, D. H., 2008. Evaluation of three polymerase chain reaction techniques for detection of *Brucella* DNA in peripheral human blood. Canadian Journal of Microbiology, 54(5), 352-357.

Baily, G., Krahn, J., Drasar, B., Stoker, N., 1992. Detection of *Brucella melitensis* and *Brucella abortus* by DNA amplification. Journal of Tropical Medicine and Hygiene, 95 (4), 271-275.

Bandara, A. B., Contreras, A., Contreras-Rodriguez, A., Martins, A. M., Dobrean, V., Poff-Reichow, S., Rajasekaran, P., Sriranganathan, N., Schurig, G. G., Boyle, S. M., 2007. *Brucella suis* urease encoded by ure1 but not ure2 is necessary for intestinal infection of BALB/c mice. BMC Microbiology, 7, 57.

Bannatyne, R. M., Jackson, M. C., Memish, Z. 1997. Rapid diagnosis of *Brucella* bacteremia by using the BACTEC 9240 system. Journal of Clinical Microbiology, 35(10), 2673-2674.

Barbuddhe, S. B., Chakurkar, E. B., Bale, M. A., Sundaram, R. N. S. and Bansode, R. B. 2004. Prevalence of brucellosis in organized dairy farms in Goa region. Indian Journal of Animal Sciences, 74(10), 1030-1031.

Barbuddhe, S. B., Kumar, P., Malika, S. V., Singh, D. K., Gupta, L. K., 2000. Seropositivity for intracellular bacterial infections among abattoir associated personnel. The Journal of Communicable Diseases, 32, 295-299.

Barquero-Calvo, E., Chaves-Olarte, E., Weiss D. S., Guzmán-Verri, C., Chacón-Díaz, C., Rucavado, A., Moriyón, I., Moreno E., 2007. *Brucella abortus* uses a stealthy strategy to avoid activation of the innate immune system during the onset of infection. PLoS One, 2(7), e631.

Barroso, E. D., Arroyo, C. I., López R. M. J., Lozano R. J. A., López L. A., 1998. The transmission of brucellosis via breast feeding. A report of 2 cases. Anales Espanola de Pediatria, 48(1), 60-62.

Basavaraj, M. C., Baragundi, M. C., Jyothi, P., Lava, R., Basavarajappa, Hanumanthappa, A, R., Chandrappa, A. R., 2011. Seroprevalence of brucellosis in Davangere, Karnataka. Journal of Clinical and Diagnostic Research, 5, 41-44.

Bayemi, P. H., Webb, E. C., Nsongka, M. V., Unger, H., Njakoi, H., 2009. Prevalence of *Brucella abortus* antibodies in serum of Holstein cattle in Cameroon. Tropical Animal Health Production, 41(2), 141-144.

Bedi, J. S., Sharma, J. K., Gill, J. P. S., Aulakh, R. S., Sharma, C. S., Gupta V. K., 2007. Seroepidemiological survey of *Brucella* agglutinins in animal handlers in Punjab, India. Indian Journal of Animal Sciences, 77(12), 1283-1285.

Beh, K. J., 1973. Distribution of *Brucella* antibody among immunoglobulin classes and a low molecular weight antibody fraction in serum and whey of cattle. Research in Veterinary Science, 14(3), 381-384.

Beh, K. J., 1974. Quantitative distribution of *Brucella* antibody amongst immunoglobulin classes in vaccinated and infected cattle. Research in Veterinary Sciences, 17(1), 1-4.

Beja-Pereira A., Bricker, B., Chen, S., Almendra, C., White, P. J., Luikart, G., 2009. DNA genotyping suggests that recent brucellosis outbreaks in the Greater Yellowstone Area originated from elk. Journal of Wildlife Diseases, 45(4), 1174-1177.

Bercovich, Z., 1998. Maintenance of *Brucella abortus*-free herds: a review with emphasis on the epidemiology and the problems in diagnosing brucellosis in areas of low prevalence. Veterinary Quarterly, 20(3), 81-88.

Blasco, J. M., 1990. *Brucella ovis.* In: Nielsen K., Duncan JR., (Editors). Animal Brucellosis. CRC Press, Boca Raton Fl.; 351-378.

Bogdanovich, T., Skurnik, M., Lübeck, P. S., Ahrens, P., Hoorfar, J., 2004. Validated 5' nuclease PCR assay for rapid identification of the genus *Brucella.* Journal of Clinical Microbiology, 42(5), 2261-2263.

Bonfoh, B., Kasymbekov, J., Dürr, S., Toktobaev, N., Doherr, M. G., Schueth, T., Zinsstag, J., Schelling, E., 2012. Representative seroprevalences of brucellosis in humans and livestock in Kyrgyzstan. EcoHealth, 9(2), 132-138.

Borba, M. R., Stevenson, M. A., Gonçalves, V. S., Neto, J. S., Ferreira, F., Amaku, M., Telles, E. O., Santana, S. S., Ferreira, J. C., Lôbo, J. R., Figueiredo, V. C., Dias, R. A., 2013. Prevalence and risk-mapping of bovine brucellosis in Maranhão State, Brazil. Preventive Veterinary Medicine, 110(2), 169-176.

Boschiroli, M. L., Ouahrani-Bettache, S., Foulongne, V., Michaux-Charachon, S., Bourg, G., Allardet-Servent, A., Cazevieille, C., Lavigne, J. P., Liautard, J. P., Ramuz, M., O'Callaghan, D., 2002. Type IV secretion and *Brucella* virulence. Veterinary Microbiology, 90(1-4), 341-348.

Boukary, A. R., Saegerman, C., Abatih, E., Fretin, D., Alambe, ´ dji Bada, R., et al., 2013. Seroprevalence and potential risk factors for *Brucella* spp. infection in traditional cattle, sheep and goats reared in urban, periurban and rural areas of Niger. PLoS One, 8, e83175.

Bourg, G., O'Callaghan, D., Boschiroli, M. L., 2007. The genomic structure of *Brucella* strains isolated from marine mammals gives clues to evolutionary history within the genus. Veterinary Microbiology, 125(3-4), 375-380.

Brahmabhatt, M. N., Varasada, R. N., Bhong, C. D., Nayak, J. B., 2009. Seroprevalence of *Brucella* spp. in buffaloes in the central Gujarat region of India. Buffalo Bulletein, 28(2), 73-75.

Braun, W., Bonestell, A. E., 1947. Independent variation of characteristics in *Brucella abortus* variants and their detection. American Journal of Veterinary Research, 8(29), 386-390.

Bricker, B. J. and Halling, S. M., 1994. Differentiation of *Brucella abortus* bv. 1, 2, and 4, *Brucella melitensis*, *Brucella ovis*, and *Brucella suis* bv.1 by PCR. Journal of Clinical Microbiology, 32(11), 2660-2666.

Bricker, B. J., 2000. Characterization of the three ribosomal RNA operons rrnA, rrnB, and rrnC, from *Brucella melitensis*. Gene, 255(1), 117-126.

Bricker, B. J., Ewalt, D. R., Halling, S. M., 2003. *Brucella* 'HOOF-Prints': strain typing by multi-locus analysis of variable number tandem repeats (VNTRs). BMC Microbiology, 3, 15.

Bricker, B. J., Ewalt, D. R., Olsen, S. C., Jensen, A. E., 2003. Evaluation of the *Brucella abortus* species-specific polymerase chain reaction assay, an improved version of the *Brucella* AMOS polymerase chain reaction assay for cattle. Journal of Veterinary Diagnostic Investigation, 15(4), 374-378.

Bricker, B. J., Halling, S. M., 1995. Enhancement of the *Brucella* AMOS PCR assay for differentiation of *Brucella abortus* vaccine strains S19 and RB51. Journal of Clinical Microbiology, 33(6), 1640–1642.

Briones, G., Iñón de Iannino, N., Roset, M., Vigliocco, A., Paulo, P. S., Ugalde, R. A., 2001. *Brucella abortus* cyclic beta-1, 2-glucan mutants have reduced virulence in mice and are defective in intracellular replication in HeLa cells. Infection and Immunity, 69(7), 4528-4535.

Brower, A., Okwumabua, O., Massengill, C., Muenks, Q., Vanderloo, P., Duster, M., Homb, K., Kurth, K. 2007. Investigation of the spread of *Brucella canis* via the U.S. Interstate dog trade. International Journal of Infectious Diseases, 11(5), 454-458.

Butler, J. E., Seawright, G. L., McGivern, P. L., Gilsdorf, M., 1986. Preliminary evidence for a diagnostic immunoglobulin G1 antibody response among culture-positive cows vaccinated with *Brucella abortus* strain 19 and challenge exposed with strain 2308. American Journal of Veterinary Research, 47(6), 1258-1264.

Buyukcangaz, E., and Sen, A., 2007. The first isolation of *Brucella melitensis* from bovine aborted fetus in Turkey. Journal of Biological and Environmental Sciences, 1, 139-142.

Buyukcangaz, E., Sen, A., Carli, K. T., Kahya, S. 2011. Comparison of direct culture versus PCR for the detection of *Brucella i*n aborted fetuses of cattle and sheep in Turkey. Veterinary Record, 168(16), 430.

Cadmus, S. I., Adesokan, H. K., Adedokun, B. O., Stack, J. A., 2010. Seroprevalence of bovine brucellosis in trade cattle slaughtered in Ibadan, Nigeria, from 2004-2006. Journal of South African Veterinary Association, 81(1), 50-53.

Cadmus, S. I., Alabi, P. I., Adesokan, H. K., Dale, E. J., Stack, J. A., 2013. Serological investigation of bovine brucellosis in three cattle production systems in Yewa Division, south-western Nigeria. Journal of the South African Veterinary Association, 84(1), E1-6.

Campero, C. M., Ladds, P. W., Hoffmann, D., Duffield, B., Watson, D., Fordyce, G., 1990. Immunopathology of experimental *Brucella abortus* strain 19 infection of the genitalia of bulls. Veterinary Immunology and Immunopathology, 24(3), 235-246.

Capasso, L., 2002. Bacteria in two-millennia-old cheese, and related epizoonoses in Roman populations. Journal of Infection, 45(2), 122-127.

Carmichael, L. E., 1990. *Brucella canis*. In: Nielsen K and Duncan JR (Editors), Animal Brucellosis. CRC Press, Boca Raton FL, 335-350.

Carvalho Neta, A. V., Mol, J. P., Xavier, M. N., Paixão, T. A., Lage, A. P. and Santos, R. L., 2010. Pathogenesis of bovine brucellosis. The Veterinary Journal, 184(2), 146-155.

Castaneda M. R., 1947. A practical method for routine blood cultures in brucellosis. Proceedings of the Society for Experimental Biology and Medicine, 64(1), 114.

Castell Monsalve, J., Gutiérrez Avila, G., Ruiz Valdepeñas, M. A., 2009. Three outbreaks of brucellosis in a one-year period investigated by the occupational health service in Ciudad Real (Spain). Gaceta Sanitaria, 23(6), 562-563.

CDC, 1994. Brucellosis outbreak at a pork-processing plant- North Carolina-1992, CDC, Morbidity and Mortality Weekly Reporter, 43 (7), 113-116.

Celebi, G., Külah, C., Kiliç, S., Ustündağ, G., 2007. Asymptomatic *Brucella* bacteraemia and isolation of *Brucella melitensis* biovar 3 from human breast milk. Scandinavian Journal of Infectious Diseases, 39(3), 205-208.

Cerekci, A., Kılıç, S., Bayraktar, M., Uyanık, M. H., Yaşar, E., Esen, B., 2011. Comparison of conventional methods and real-time multiplex polymerase chain reaction for identification and typing of *Brucella* isolates of human origin. Mikrobiyoloji Bülteni, 45(3), 392-400.

Cetinkaya, Z., Aktepe, O. C., Ciftci, I. H., Demirel, R., 2005. Seroprevalence of human brucellosis in a rural area of Western Anatolia, Turkey. Journal of Health Population and Nutrition, 23(2), 137-141.

Chahota, R., Sharma, M., Katoch R. C., Verma S., Singh, M. M., Kapoor, V., Asrani, R. K., 2003. Brucellosis outbreak in an organized dairy farm involving cows and in contact human beings, in Himachal Pradesh, India. Veterinarski Arhive, 73(2), 95-102.

Chand, P. and Chhabra, R., 2013. Herd and individual animal prevalence of bovine brucellosis with associated risk factors on dairy farms in Haryana and Punjab in India. Tropical Animal Health Production, 45(6), 1313-1319.

Chikweto, A., Tiwari, K., Kumthekar, S., Stone, D., Louison, B., Thomas, D., Sharma, R., Hariharan, H., 2013. Serologic detection of antibodies to *Brucella* spp. using a commercial ELISA in cattle in Grenada, West Indies. Tropical Biomedicine, 30(2), 277-280.

Cockerill, F. R. 3rd, Reed, G. S., Hughes, J. G., Torgerson, C. A., Vetter, E. A., Harmsen, W. S., Dale, J. C., Roberts, G. D., Ilstrup, D. M., Henry, N. K., 1997. Clinical comparison of BACTEC 9240 plus aerobic/F resin bottles and the isolator aerobic culture system for detection of bloodstream infections. Journal of Clinical Microbiology, 35(6), 1469-1472.

Colmenero, J. D., Queipo-Ortuño, M. I., Reguera, J. M., Baeza, G., Salazar, J. A., Morata, P., 2005. Real time polymerase chain reaction: a new powerful tool for the diagnosis of neurobrucellosis. Journal of Neurology, Neurosurgery and Psychiatry, 76(7), 1025-1027.

Colmenero, J. D., Reguera, J. M., Martos, F., Sánchez-De-Mora, D., Delgado, M., Causse, M., Martín-Farfán, A., Juárez, C., 1996. Complications associated with *Brucella melitensis* infection: a study of 530 cases. Medicine (Baltimore), 75(4), 195-211.

Comerci, D. J., Martinez-Lorenzo, M. J., Sieira, R., Gorvel, J. P., Ugalde R. A., 2001. Essential role of the VirB machinery in the maturation of the *Brucella abortus* - containing vacuole. Cellular Microbiology, 3, 159-168.

Cooper, C. W., 1992. Prevalence of antibody to *Brucella* in asymptomatic well individuals in Saudi Arabia. Journal of Tropical Medicine and Hygiene, 95(2), 140-142.

Corbel, M. J. (Editor), 2006. Brucellosis in humans and animals. World Health Organization, Geneva , Switzerland.

Corbel, M. J., 1972. The effect of passively acquired antibodies to *Brucella abortus* on the immune response to strain 19 vaccine. British Veterinary Journal, 128(9), xlvii-l.

Corbel, M. J., 1985. Comparison of *Brucella abortus* and *B. melitensis* antigens for the Rose Bengal plate test on sera from cattle infected with *B abortus* biovar-5. Veterinary Record, 117(15), 385-386.

Corbel, M. J., 1997. Brucellosis: an overview. Emerging Infectious Diseases, 3(2), 213-221.

Crasta, O. R., Folkerts, O., Fei, Z., Mane, S. P., Evans, C., et al., 2008. Genome Sequence of *Brucella abortus* Vaccine Strain S19 Compared to Virulent Strains Yields Candidate Virulence Genes. PLoS One, 3(5), e2193.

Crawford, R. P., Huber, J. D. and Adams, B. S., 1990. Epidemiology and surveillance. In: Nielsen, K., Duncan, J. R. (Editors): Animal Brucellosis. CRC Press, Boca Raton, 131-151.

Cutler, S. J., Whatmore, A. M. Commander, N. J., 2005. Brucellosis- new aspects of an old disease. Journal of Applied Microbiology, 98(6), 1270-1281.

D'Anastasio, R., Staniscia, T., Milia, M. L., Manzoli, L., Capasso, L., 2011. Origin, evolution and paleoepidemiology of brucellosis. Epidemiology and Infection, 139(1), 149-156.

D'Anastasio, R., Zipfel, B., Moggi-Cecchi, J., Stanyon, R., Capasso, L., 2009. Possible brucellosis in an early hominin skeleton from sterkfontein, South Africa. PLoS One, 4(7), e6439.

Dawson, C. E., Stubberfield, E. J., Perrett, L. L., King, A. C., Whatmore, A. M., Bashiruddin, J. B,,Stack, J. A., Macmillan, A. P., 2008. Phenotypic and molecular characterization of *Brucella* isolates from marine mammals. BMC Microbiology, 8, 224.

Debeaumont, C., Falconnet, P. A., Maurin, M., 2005. Real-time PCR for detection of *Brucella* spp. DNA in human serum samples. European Journal of Clinical Microbiology and Infectious Diseases, 24(12), 842-845.

Deepak, S., Bronson, S. G., Sibi, Joseph, W., Thomas, M., 2003. *Brucella* isolated from bone marrow. Journal of the Association of Physicians of India, 51,717-718.

Deepthy, B. J., Sreejit, K., Jisha, P., Ravindran, P.C., 2013. Sero epidemiology of brucellosis among high risk occupational groups by conventional methods and indirect enzyme linked immunosorbent assay International Journal of Current Research, 5 (10), 3195-3198.

DelVecchio, V. G., Kapatral, V., Redkar, R. J., Patra, G., Mujer, C., Los, T., Ivanova, N., Anderson, I., Bhattacharyya, A., Lykidis, A., Reznik, G., Jablonski, L., Larsen, N. et al., 2002. The genome sequence of the facultative intracellular pathogen *Brucella melitensis.* Proceedings of the National Academy of Sciences U. S. A., 99(1), 443-448.

Dembek, and Zygmunt. F. (Editors), 2007. Medical aspects of biological warfare. Washington D, C. Borden Institute, Chapter 9, pp 185-198.

Derakhshandeh, A., Firouzi, R., Goudarztalejerd, A., 2013. Detection of virulence genes (*bvf A, vir B and ure*) in *Brucella melitensis* isolated from aborted fetuses of sheep and goats. Iranian Journal of Microbiology, 5(4), 402-405.

Dhand, N. K., Gumber, S., Singh, B. B., Aradhana Bali, M. S., Kumar, H., Sharma, D. R., Singh, J., Sandhu, K. S., 2005. A study on the epidemiology of brucellosis in Punjab (India) using Survey Toolbox. Revue Scientifique Et Technique Office International des Epizooties, 24(3), 879-885.

Díaz, R., Casanova, A., Ariza, J., Moriyón, I., 2011. The Rose Bengal Test in human brucellosis: a neglected test for the diagnosis of a neglected disease. PLoS Neglected Tropical Diseases, 5(4), e950.

Doern, G. V., 2000. Detection of selected fastidious bacteria. Clinical Infectious Diseases, 30(1), 166-173.

Doganay, M. and Aygen, B., 2003. Human brucellosis: an overview. International journal of infectious diseases, 7(3), 173-182.

Dorneles, E. M., Freire, G. N., Dasso, M. G., Poester, F. P., Lage, A. P., 2014b. Genetic diversity of *Brucella ovis* isolates from Rio Grande do Sul, Brazil, by MLVA16. BMC Research Notes, 7, 447.

Dorneles, E. M., Santana, J. A., Alves, T. M., Pauletti, R. B., Mol, J. P., Heinemann, M. B., Lage, A. P., 2014a. Genetic stability of *Brucella abortus* isolates from an outbreak by multiple-locus variable-number tandem repeat analysis (MLVA16). BMC Microbiology, 14, 186.

Dossey, B. M., 2010. Florence Nightingale: her Crimean fever and chronic illness. Journal of Holistic Nursing, 28(1), 38-53.

Durmaz, G., Us, T., Aydinli, A., Kiremitci, A., Kiraz, N., Akgün, Y., 2003. Optimum detection times for bacteria and yeast species with the BACTEC 9120 aerobic blood culture system: evaluation for a 5-year period in a Turkish university hospital. Journal of Clinical Microbiology, 41(2), 819-821

Earhart, K., Vafakolov, S., Yarmohamedova, N., Michael, A., Tjaden, J., Soliman, A., 2009. Risk factors for brucellosis in Samarqand Oblast, Uzbekistan. International Journal of Infectious Diseases, 13(6), 749-753.

Edmonds, M. D., Cloeckaert, A., Booth, N. J., Fulton, W. T., Hagius, S. D., Walker, J. V., Elzer, P. H. 2001. Attenuation of a *Brucella abortus* mutant lacking a major 25 kDa outer membrane protein in cattle. American Journal of Veterinary Research, 62(9), 1461-1466.

Eisenberg, T., Hamann, H. P., Kaim, U., Schlez, K., Seeger, H., Schauerte, N., Melzer, F., Tomaso, H., Scholz, H. C., Koylass, M. S., Whatmore, A. M., Zschöck, M., 2012.

Isolation of potentially novel *Brucella* spp. from frogs. Applied Environmental Microbiology, 78(10), 3753-3755.

Escobar, G. I., Jacob, N. R., López, G., Ayala, S. M., Whatmore, A. M., Lucero, N. E., 2013. Human brucellosis at a pig slaughterhouse. Comparative Immunology Microbiology and Infectious Diseases, 36(6), 575-580.

Esmaeili S., Pourhossein, B., Gouya, M. M., Amiri, F. B., Mostafavi, E., 2014. Seroepidemiological survey of Q fever and brucellosis in Kurdistan province, western Iran. Vector Borne Zoonotic Diseases, 14(1), 41-45.

Etemadi, H., Raissadat, A., Pickett, M. J., Zafari, Y., Vahedifar, P. 1984. Isolation of *Brucella* spp. from clinical specimens. Journal of Clinical Microbiology, 20(3), 586.

Evans, A. S., Brachman, P. S., 2009. Bacterial infections of human: epidemiology and control. (4thedn), Springer Science Business Media, LLC, 233, New York, NY 10013, USA.

Ewalt, D. R., 1989. Comparison of three culture techniques for the isolation of *Brucella abortus* from bovine supramammary lymph nodes. Journal of Veterinary Diagnostic Investigation, 1(3), 227-230.

FAO/ WHO, 1986. Joint WHO/FAO expert committee on brucellosis. 6th report. WHO Techical Report Series, 740, 1-132.

Farina, F., Fuser, R., Rossi, M., Scotton, P.G., 2008. Brucellosis outbreak in Treviso province caused by infected cheese from an endemic area. Le Infezioni in Medicina, 16(3), 154-157.

Farrell, I. D., 1974. The development of a new selective medium for the isolation of *Brucella abortus* from contaminated sources. Research in Veterinary Science, 16(3), 280-286.

Fekete, A., Bantle, J. A., Halling, S. M., Stich, R. W., 1992. Amplification fragment length polymorphism in *Brucella* strains by use of polymerase chain reaction with arbitrary primers. Journal of Bacteriology,174(23), 7778-7783.

Fernandez-Prada, C. M., Nikolich, M., Vemulapalli, R., Sriranganathan, N., Boyle, S. M., Schurig, G. G., Hadfield, T. L., Hoover, D. L., 2001. Deletion of wboA enhances activation of the lectin pathway of complement in *Brucella abortus* and *Brucella melitensis*. Infection and Immunity, 69(7), 4407-4416.

Ferreira, A. C., Chambel, L., Tenreiro, T., Cardoso, R., Flor, L., et al., 2012. MLVA16 typing of Portuguese human and animal *Brucella melitensis* and *Brucella abortus* isolates. PLoS One, 7(8), e42514.

Forbes, L. B., Steele, T. B. 1989. An outbreak of *Brucella abortus* biovar 2 in Canadian cattle. Canadian Veterinary Journal, 30(11), 888-893.

Foster, G., Jahans, K. L., Reid, R. J., Ross, H. M., 1996. Isolation of *Brucella* species from cetaceans, seals and an otter. Veterinary Record, 138(24), 583-586.

Foster, G., Osterman, B. S., Godfroid. J., Jacques, I., Cloeckaert, A., 2007. *Brucella ceti* sp. nov. and *Brucella pinnipedialis* sp. nov. for *Brucella* strains with cetaceans and seals as their preferred hosts. International Journal of Systematic and Evolutionary Microbiology, 57(Pt 11), 2688-2693.

Foulongne, V., Bourg, G., Cazevieille, C., Michaux Charachon, S., O'Callaghan, D., 2000. Identification of *Brucella suis* genes affecting intracellular survival in an in vitro human macrophage infection model by signature- tagged transposon mutagenesis. Infection and Immunity, 68(3), 1297-1303.

Franco, M. P., Mulder, M., Gilman, R. H., Smits, H. L., 2007. Human brucellosis. The Lancet Infectious Diseases, 7(12), 775-786.

Fretin, D., Mori, M., Czaplicki, G., Quinet, C., Maquet, B., Godfroid, J., Saegerman, C., 2013. Unexpected *Brucella suis* biovar 2 Infection in a dairy cow, Belgium. Emerging Infectious Diseases Journal, 19(12), 2053-2054.

Galbraith, N. S., Ross, M. S., de Mowbray. R. R., Payne. D. J., 1969. Outbreak of *Brucella melitensis* type 2 infection in London. British Medical Journal, 1(5644), 612-614.

Gall, D., Nielsen, K., 2004. Serological diagnosis of bovine brucellosis: a review of test performance and cost comparison Revue Scientifique Et Technique Office International des Epizooties, 23(3), 989-1002.

Gallien, P., Dorn, C., Alban, G., Staak, C., Protz, D., 1998. Detection of *Brucella* species in organs of naturally infected cattle by polymerase chain reaction. Veterinary Record, 142 (19), 512-514.

Ganado, W., Bannister, W., 1960. Bacteremia in human brucellosis. British Medical Journal, 1, 602-603.

García-Yoldi, D., Marín, C. M., De Miguel, M. J., Muñoz, P. M., Vizmanos, J. L., López-Goñi I., 2006. Multiplex PCR assay for the identification and differentiation of all *Brucella* species and the vaccine strains *Brucella abortus* S19 and RB51 and *Brucella melitensis* Rev1. Clinical Chemistry, 52**,** 779-781.

Garin-Bastuji, B., 1993. Brucelloses bovine, ovine et caprine: Contrôle et prevention. Le point Veterinaire 25, 107-114.

Garshasbi, M., Ramazani, A., Sorouri, R., Javani, S., Moradi, S., 2014. Molecular detection of *Brucella* species in patients suspicious of Brucellosis from Zanjan, Iran. Brazilian Journal of Microbiology, 45(2), 533-538.

Ghodasara, S., Roy, A., Rank, D. N., Bhanderi, B. B., 2010. Identification of *Brucella* spp. from animals with reproductive disorders by polymerase chain reaction assay. Buffalo Bulletin, 29(2), 98.

Ghodsara, S. and Roy, A., 2012. Isolation, identification and biochemical characterization of *Brucella* from reproductive disorders of animals. Indian Journal of Comparative Microbiology Immunology and Infectious Diseases, 33(1-2), 34-40.

Giannacopoulos, I., Eliopoulou, M. I., Ziambaras, T., Papanastasiou, D. A., 2002. Transplacentally transmitted congenital brucellosis due to *Brucella abortus*. Journal of Infection, 45(3), 209-210.

Glocwicz, J., Stonecipher, S., Schulte, J., 2010. Maternal and congenital brucellosis in Texas: changing travel patterns and laboratory implications. Journal of Immigrant and Minority Health, 12(6), 952-955.

Godfroid, J., 2002. Brucellosis in wildlife. Revue Scientifique Et Technique Office International des Epizooties, 21(2), 277-286.

Godfroid, J., Cloeckaert, A., Liautard, J., 2005. From the discovery of the Malta fever's agent to the discovery of a marine mammal reservoir, brucellosis has continuously been a re-emerging zoonosis. Veterinary Research, 36(3), 313-326.

Godfroid, J., Nielsen, K., Saegerman, C., 2010. Diagnosis of Brucellosis in Livestock and Wildlife. Croatian Medical Journal, 51(4), 296-305.

Gómez, M. C., Nieto, J. A., Rosa, C., Geijo, P., Escribano, M. A., Muñoz, A., López, C., 2008. Evaluation of seven tests for diagnosis of human brucellosis in an area where the disease is endemic. Clinical and Vaccine Immunology, 15(6), 1031-1033.

Gomo, C., de Garine-Wichatitsky, M., Caron, A., Pfukenyi, D. M., 2012. Survey of brucellosis at the wildlife-livestock interface on the Zimbabwean side of the Great

Limpopo Transfrontier Conservation Area. Tropical Animal Health Production, 44(1), 77-85.

Gomo, C., Musari, S., De Garine-Wichatitsky, M., Caron, A., Pfukenyi, D. M., Van Heerden, H., 2012. Detection of *Brucella abortus* in Chiredzi district in Zimbabwe. Onderstepoort Journal of Veterinary Research, 79(1), E1-5.

Grace, D., Mutua, F., Ochungo, P., Kruska, R., Jones, K., Brierley, L., Lapar, L., Said, M., Herrero, M., Phuc, P.M., Thao, N.B., Akuku, I., Ogutu, F. 2012. Mapping of poverty and likely zoonoses hotspots. Zoonoses Project 4. Report to the UK Department for International Development. International Livestock Research Institute, Nairobi, Kenya.

Greenfield, R. A., Drevets, D. A., Machado, L. J., Voskuh,l G. W, Cornea, P. and Bronze, M. S., 2002. Bacterial pathogens as biological weapons and agents of bioterrorism. American Journal of Medical Science, 323, 299-315.

Gumi, B., Firdessa, R., Yamuah, L., Sori, T., Tolosa, T., Aseffa, A., Zinsstag, J., Schelling, E., 2013. Seroprevalence of brucellosis and Q-Fever in southeast ethiopian pastoral livestock. Journal of Veterinary Science Medical Diagnosis, 2(1), doi: 10.4172/2325-9590.1000109.

Gür, A., Geyik, M. F., Dikici, B., Nas, K., Cevik, R., Sarac, J., Hosoglu, S., 2003. Complications of brucellosis in different age groups: a study of 283 cases in southeastern Anatolia of Turkey. Yonsei Medical Journal. 44(1), 33-44.

Guzmán-Verri, C., Albert, D., Garin-Bastuji, B., 2011. New Bruce-ladder multiplex PCR assay for the biovar typing of *Brucella suis* and the discrimination of *Brucella suis* and *Brucella canis*. Veterinary Microbiology, 154(1-2), 152-155.

Guzmán-Verri, C., Chaves-Olarte, E., von Eichel-Streiber, C., López-Goñi, I., Thelestam, M., Arvidson, S., Gorvel, J. P., Moreno, E., 2001. GTPases of the Rho subfamily are required for *Brucella abortus* internalization in nonprofessional phagocytes: direct activation of Cdc42. The Journal of Biological Chemistry, 276(48), 44435-44443.

Gwida, M., Dahouk, S., Melzer, F., Rösler, U., Neubauer, H., 2010. Brucellosis–Regionally emerging zoonotic disease? Croatian Medical Journal, 51(4), 289-295.

Gyuranecz, M., Szeredi, L., Rónai, Z., Dénes, B., Dencso, L., Dán, Á., Pálmai, N., Hauser, Z., Lami, E., Makrai, L., Erdélyi, K., Jánosi, S., 2011. Detection of *Brucella canis*-induced reproductive diseases in a kennel. Journal of Veterinary Diagnostic Investigation, 23(1), 143-147.

Hall, W. H., 1990. Modern chemotherapy for brucellosis in humans. Reviews of Infectious Diseases, 12(6), 1060-1099.

Hamdy, M. E. and Amin A. S., 2002. Detection of *Brucella* species in the milk of infected cattle, sheep, goats and camels by PCR. The Veterinary Journal, 163(3), 299-305.

Handa, R., Singh, S., Singh, N., Wali, J. P., 1998. Brucellosis in north India: results of a prospective study. Journal of Communicable Diseases, 30(2), 85-87.

Hayes, M., Kilroy, A., Ashe, S., Power, S., Kenny, K., Collins, D. M., & More, S. J., 2010. Outbreak of bovine brucellosis in County Clare, Ireland, in 2005.The Veterinary record, 166(4), 107-111.

Hekmatimoghaddam, S., Sadeh M., Khalili, M. B., Mollaabedin, M., Sazmand, A., 2013. Comparison of PCR, Wright agglutination test and blood culture for diagnosis of brucellosis in suspected patients. Pakistan Journal of Biological Sciences, 16(22), 1589-1592.

Hénault, S., Calvez, D., Thiébaud, M., Bouličre, M., Garin-Bastuji, B., 2000. Validation of a nested-PCR based on the *IS6501/711* sequence for the detection of *Brucella* in animal samples. Proceedings of the Brucellosis 2000 International Research Conference 9I including the 53rd Brucelllosis Reserach Conference. Nimes, France.

Her, M., Cho, D. H., Kang, S. I., Lim, J. S., Kim, H. J., Cho, Y. S., Hwang, I. Y., Lee, T., Jung, S. C., Yoo, H. S., 2010. Outbreak of brucellosis in domestic elk in Korea. Zoonoses and Public Health, 57(3), 155-161.

Hesterberg, U.W., Bagnall, R., Perrett, K., Bosch, B., Horner, R., Gummow, B., 2008. A serological prevalence survey of *Brucella abortus* in cattle of rural communities in the province of KwaZulu- natal, South Africa. Journal of South African Veterinary Association, 79(1), 15-18.

Hinić, V., Brodard, I., Thomann, A., Cvetnić, Z., Makaya, P. V., Frey, J., Abril, C., 2008. Novel identification and differentiation of *Brucella melitensis*, *B. abortus*, *B. suis*, *B. ovis*, *B. canis*, and *B. neotomae* suitable for both conventional and real-time PCR systems. Journal of Microbiological Methods, 75(2), 375-378

Hirsh, D. C., Zee, Y. C., 2003. Microbiologia Veterinária. Rio de Janeiro: Guanabara Koogan. pp. 191-195.

Hong, P. C., Tsolis, R. M., Ficht, T. A. 2000. Identification of genes required for chronic persistence of *Brucella abortus* in mice. Infection and Immunity, 68(7), 4102-4107.

Huber, B., Scholz, H. C., Lucero, N., Busse, H. J., 2009. Development of a PCR assay for typing and subtyping of *Brucella* species. International Journal of Medical Microbiology, 299(8), 563-573.

Huber, J. D., Nicoletti, P. 1986. Comparison of the results of card, rivanol, complement-fixation, and milk ring tests with the isolation rate of *Brucella abortus* from cattle. American Journal of Veterinary Research, 47(7), 1529-1531.

Hunter, D., and Allen, J., 1972. An evaluation of milk and blood tests used to diagnose brucellosis. Veterinary Record, 91(13), 310-312.

Ibrahim, N., Belihu, K., Lobago, F., Bekana, M., 2010. Sero-prevalence of bovine brucellosis and its risk factors in Jimma zone of Oromia Region, South-western Ethiopia. Tropical Animal Health Production, 42(1), 35-40.

Imaoka, K., Kimura, M., Suzuki, M., Kamiyama, T., Yamada, A., 2007. Simultaneous detection of the genus *Brucella* by combinatorial PCR. Japenese Journal of Infectious Diseases, 60(2-3), 137-139.

Işeri, S., Bulut, C., Yetkin, M. A., Kinikli, S., Demiröz, A. P., Tülek, N., 2006. Comparison of the diagnostic value of blood and bone marrow cultures in brucellosis. Mikrobiyoloji Bülteni, 40(3), 201-206.

Islam, M. A., Khatun, M. M., Were, S. R., Sriranganathan, N., Boyle, S. M., 2013. A review of *Brucella* seroprevalence among humans and animals in Bangladesh with special emphasis on epidemiology, risk factors and control opportunities. Veterinary Microbiology, 166(3-4), 317-326.

Isloor, S., Renukaradhya, G. J., Rajasekhar, M. A., 1998. A serological survey of bovine brucellosis in India. Revue Scientifique Et Technique Office International des Epizooties, 17(3), 781-785.

Jagapur, R. V., Rathore, R., Karthik, K. and Somavanshi R., 2013. Seroprevalence studies of bovine brucellosis using indirect-enzyme-linked immunosorbent assay (i-ELISA) at organized and unorganized farms in three different states of India, Veterinary World, 6(8), 550-553.

Jain, R., Tilak, V., 2008. Evaluation of different serological techniques in laboratory diagnosis of brucellosis. Journal of Indian Medical Association, 106(8),520-524.

Jain, U., Bist, B., Sahzad, Pragati, and Dwivedi, K. 2013. Outbreak of brucellosis in buffaloes aborted in village Mahuan, district Mainpuri, U.P., India- A case report, Veterinary World, 6(1), 51-52.

Jama'ayah, M. Z., Heu, J. Y., Norazah, A., 2011. Seroprevalance of brucellosis among suspected cases in Malaysia. The Malaysian Journal of Pathology, 33(1), 31-34.

Jensen, A. E., Cheville, N. F., Ewalt, D. R., Payeur, J. B., Thoen, C. O., 1995. Application of pulsed-field gel electrophoresis for differentiation of vaccine strain RB51 from field isolates of *Brucella abortus* from cattle, bison, and elk. American Journal of Veterinary Research, 56(3), 308-312.

Jensen, A. E., Cheville, N. F., Thoen, C. O., MacMillan, A. P., Miller, W. G., 1999. Genomic fingerprinting and development of a dendrogram for *Brucella* spp. isolated from seals, porpoises, and dolphins. Journal of Veterinary Diagnostic Investigation, 11(2), 152-157.

John, K., Fitzpatrick, J., French, N., Kazwala, R., Kambarage, D., Mfinanga, G. S., MacMillan A., Cleaveland. , S., 2010. Quantifying risk factors for human brucellosis in rural northern Tanzania. PLoS One, 5(4), e9968.

Kadri, S. M., Rukhsana, A., Laharwal, M. A., Tanvir, M. 2000. Seroprevalence of brucellosis in Kashmir (India) among patients with pyrexia of unknown origin. Journal of Indian Medical Association, 98,170-171.

Kalla, A., Chadda, V. S., Gauri, L. A., Gupta, A., Jain, S., Gupta, B. K., Chaddha, S., Nayak, K. C., Singh, V. B., Kumhar, M. R., 2001. Outbreak of polyarthritis with pyrexia in Western Rajasthan. Journal of the Assocociation of Physicians of India, 49,963-965.

Kaltungo, B. Y., Saidu, S. N., Sackey, A. K., Kazeem, H. M., 2013. Serological evidence of brucellosis in goats in Kaduna north senatorial district of Kaduna state, Nigeria. ISRN Veterinary Science, 2013, 963673.

Karabay, O., Sencan, I., Kayas, D., Sahin, I., 2004. Ofloxacin plus rifampicin versus doxycycline plus rifampicin in the treatment of brucellosis: a randomized clinical trial [ISRCTN11871179]. BMC Infectious Diseases, 4, 18.

Karagiannis, I., Mellou, K., Gkolfinopoulou, K., Dougas, G., Theocharopoulos, G., Vourvidis, D., Ellinas, D., Sotolidou, M., Papadimitriou, T., Vorou, R., 2012. Outbreak investigation of brucellosis in Thassos, Greece- 2008. Eurosurveillance, 17(11), pii: 20116.

Karplus, R., Ramlawi, A., Banai, M., Maayan, S. 2007. The use of ELISA in a seroprevalence study of *Brucella* antibodies in West Bank Palestinian women of childbearing age. International Journal of Infectious Diseases, 11(4), 367-368.

Kashiwazaki, Y., Ecewu, E., Imaligat, J. O., Mawejje, R., Kirunda, M., Kato, M., Musoke, G. M., Ademun, R. A., 2012. Epidemiology of bovine brucellosis by a combination of rose bengal test and indirect ELISA in the five districts of Uganda. Journal of Veterinary Medical Science, 74(11), 1417-1422.

Kassahun, J., Yimer, E., Geyid, A., Abebe, P., Newayeselassie, B., Zewdie, B., Beyene. M., Bekele, A., 2006. Sero-prevalence of brucellosis in occupationally exposed people in Addis Ababa, Ethiopia. Ethiopian Medical Journal, 44(3), 245-252.

Kattar, M. M., Zalloua, P. A., Araj, G. F., Samaha-Kfoury, J., Shbaklo, H., Kanj, S. S., Khalife, S., Deeb, M., 2007. Development and evaluation of real-time polymerase chain reaction assays on whole blood and paraffin-embedded tissues for rapid diagnosis of human brucellosis. Diagnostic Microbiology and Infectious Disases, 59(1), 23-32.

Kaur, P., Sharma, N. S., Jand, S. K., Oberoi, M. S., 2006. Isolation and identification of *Brucella abortus* from aborted cattle and buffaloes and evaluation of their antibiogram. Indian Journal of Animal Sciences, 76(2), 105-108.

Kay, G. L., Sergeant, M. J., Giuffra, V., Bandiera, P., Milanese, M., Bramanti, B., Bianucci, R., Pallen, M. J., 2014. Recovery of a medieval *Brucella melitensis* genome using shotgun metagenomics. mBio, 5(4), e1337-14.

Keid, L. B., Soares, R. M., Vieira, N. R., Megid, J., Salgado, V. R., Vasconcellos, S. A., da Costa, M., Gregori, F., Richtzenhain, L. J., 2007. Diagnosis of canine brucellosis: comparison between serological and microbiological tests and a PCR based on primers to 16S-23S rDNA interspacer. Veterinary Research Communication, 31(8), 951-965.

Keppie, J., Williams, A. E., Witt, K., Smith, H., 1965. The role of erythritol in the tissue localization of the brucellae. British journal of Experimental Pathology, 46(1), 104-108.

Khajuria, B. K., Malik, M. A., Tiwari, A., Sharma, N., Wazir, V. S., 2014. Seroprevalence Studies of Brucellosis at organized and unorganized cattle farms in north India. International Journal of Agriculture, Environment and Biotechnology, 7(3), 499-505.

Kochar, D. K., Gupta, B. K., Gupta, A., Kalla, A., Nayak, K. C., Purohit, S. K. 2007. Hospital based case series of 175 cases of serologically confirmed brucellosis in Bikaner. Journal of the Association of Physicians of India, 55, 271-275.

Kollannur, J. D., Rathore, R., Chauhan, R. S., Aland, A. (2007). Epidemiology and economics of brucellosis in animals and its zoonotic significance. In Animal health, animal welfare and biosecurity. Proceedings of 13th International Congress in Animal Hygiene, Tartu, Estonia, 17-21 June, 2007. volume 1. (pp. 466-468). Estonian University of Life Sciences, Jõgeva Plant Breeding Institute, Estonian Research Institute of Agriculture.

Kubuafor, D. K., Awumbila, B., Akanmori, B. D., 2000. Seroprevalence of brucellosis in cattle and humans in the Akwapim-South district of Ghana: public health implications. Acta Tropica, 76(1), 45-48.

Kumar, A., Kumar, A., Sadish, S., Latha, C., Kumar, K., Kumar, A., 2010. Epidemiology of brucellosis in occupationally exposed human beings. Indian Journal of Animal Research, 44, 188-192.

Kumar, P., Singh, D. K., Barbuddhe, S. B., 1997. Sero-prevalence of brucellosis among abattoir personnel of Delhi. Journal of Communicable Diseases, 29(2), 131-137.

Kumaraswamy, P. L. S., Nainar, P., Somasundaran, N., 2015. Detection of *Brucella* immunoglobulin G among high risk group: Significance in the diagnosis and follow-up. Journal of the Scientific Society, 42(1), 21-23.

Kutlu, M., Ergonul, O., Sayin-Kutlu, S., Guven, T., Ustun, C., Alp-Cavus, S., Ozturk, S. B., Acicbe, O., Akalin, S., Tekin, R., Tekin-Koruk, S., Demiroglu, Y. Z., Keskiner, R., Gönen, I., Sapmaz-Karabag, S., Bosnak, V., Kazak, E., 2014. Risk factors for occupational brucellosis among veterinary personnel in Turkey. Preventive Veterinary Medicine, 117(1), 52-58.

Kuzdas, C. D., Morse, E. V., 1953. A selective medium for the isolation of brucellae from contaminated materials. Journal of Bacteriology, 66(4), 502-504.

Lamb, V. L., Jones, L. M., Schurig, G. G., Berman, D. T., 1979. Enzyme-linked immunosorbent assay for bovine immunoglobulin subclass-specific response to *Brucella abortus* lipopolysaccharides. Infection and Immunity, 26(1), 240-247.

Lambert, G., Manthei, C. A., Deyoe, B. L., 1963. Studies on *Brucella abortus* infection in bulls. American Journal of Veterinary Reseach, 24, 1152-1157.

Langoni, H., Ichihara, S. M., Silva, A. V. D., Pardo, R. B., Tonin, F. B., Mendonça, L. J. P., Machado, J. A. D., 2000. Isolation of *Brucella* spp. from milk of brucellosis positive cows in São Paulo and Minas Gerais states. Brazilian Journal of Veterinary Research and Animal Science, 37(6), 444-448.

Lapaque, N., Moriyon, I., Moreno, E., Gorvel, J. P., 2005. *Brucella* lipopolysaccharide acts as a virulence factor. Current Opinion in Microbiology, 8(1), 60-66.

Lavigne, J.-P., Patey, G., Sangari, F. J., Bourg, G., Ramuz, M., O'Callaghan, D., Michaux-Charachon, S., 2005. Identification of a new virulence factor, Bvf A, in *Brucella suis*. Infection and Immunity, 73(9), 5524-5529.

Le Flèche, P., Jacques, I., Grayon, M., Al Dahouk, S., Bouchon, P., Denoeud, F., Nöckler, K., Neubauer, H., Guilloteau, L. A., Vergnaud, G., 2006. Evaluation and selection of tandem repeat loci for a *Brucella* MLVA typing assay. BMC Microbiology, 6(1), 9.

Leal-Klevezas, D. S., López-Merino, A., Martínez-Soriano, J. P., 1995. Molecular detection of *Brucella* spp.: rapid identification of *B. abortus* biovar I using PCR. Archives of Medical Research, 26(3), 263-267.

Leal-Klevezas, D. S., Martínez-Vázquez, I. O., García-Cantú, J., López-Merino, A., Martínez-Soriano, J. P., 2000. Use of polymerase chain reaction to detect *Brucella abortus* biovar 1 in infected goats. Veterinary Microbiology, 75(1), 91-97.

Leal-Klevezas, D. S., Martínez-Vázquez, I. O., López-Merino, A., Martínez-Soriano, J. P., 1995. Single-step PCR for detection of *Brucella* spp. from blood and milk of infected animals. Journal of Clinical Microbiology, 33(12), 3087-3090.

Lee, K., Lim, H. S., Park, W. W., Kim, S. H., Lee, D. Y., Park, M. Y., Hur, Y., 2007. Seroprevalence of brucellosis among risk population in Gyeongsangbuk-do, 2006. Journal of Preventive Medicine and Public Health, 40(4), 285-290.

Levieux, D., 1978. Bovine immunoglobulins and brucellosis. 3. Activity of IgG, IgG2 and IgM versus different commercial batches of Rose Bengal antigen. Annales de Recherches Veterinaires (Paris), 9(3), 489-493.

Li, Z. J., Cui, B. Y., Chen, H., Chen, J. D., Zhao, H. Y., Piao, D. R., Jiang, H., Zhang, L., Tang, X., Ke, C. W., Yao, Z., Tian, G. Z., 2013. Molecular typing of *Brucella suis* collected from 1960s to 2010s in China by MLVA and PFGE. Biomedical and Environmental Sciences, 26(6), 504-508.

Lindahl E., Sattorov N., Boqvist S., Sattori I., Magnusson U., 2014. Seropositivity and risk factors for *Brucella* in dairy cows in urban and peri-urban small-scale farming in Tajikistan. Tropical Animal Health Production, 46(3), 563-569.

Liu, D. (Editor). (2009). Molecular detection of foodborne pathogens. CRC Press.

Londhe, S. P., Bannalikar, A. S. and Dighe, V. D., 2011. Sero-detection of bovine brucellosis by RBPT and AB-ELISA. Animal Science Reporter, 5, 69-73.

López-Goñi, I., García-Yoldi, D., Marín, C. M., de Miguel, M. J., Muñoz, P. M., Blasco, J. M., Jacques, I., Grayon, M., Cloeckaert, A., Ferreira, A. C., Cardoso, R., Corrêa de Sá, M. I., Walravens, K., Albert, D., Garin-Bastuji, B., 2008. Evaluation of a multiplex PCR assay (Bruce-ladder) for molecular typing of all *Brucella* species, including the vaccine strains. Journal of Clinical Microbiology, 46(10), 3484-3487.

López-Goñi, I., García-Yoldi, D., Marín, C. M., de Miguel, M. J., Barquero-Calvo, E., Guzmán-Verri, C., Albert, D., Garin-Bastuji, B., 2011. New Bruce-ladder multiplex PCR assay for the biovar typing of *Brucella suis* and the discrimination of *Brucella suis* and *Brucella canis*. Veterinary Microbiology, 154(1-2), 152-155.

López-Goñi, I., Guzmán-Verri, C., Manterola, L., Sola-Landa, A., Moriyón, I., Moreno, E., 2002. Regulation of *Brucella* virulence by the two-component system BvrR/BvrS. Veterinary Microbiology, 90(1-4), 329-339.

Luce, R., Snow, J., Gross, D., Murphy, T., Grandpre, J., Daley, W. R., Brudvig, J. M., Ari, M. D., Harris, L., Clark, T, A., 2012. Brucellosis seroprevalence among workers in at-risk professions: northwestern Wyoming, 2005 to 2006. Journal of Occupational and Environmental Medicine, 54(12), 1557-60.

Lucero, N, E,, Corazza, R., Almuzara, M. N., Reynes, E., Escobar, G. I., Boeri, E., Ayala, S. M. 2010. Human *Brucella canis* outbreak linked to infection in dogs. Epidemiology and Infection, 138(2), 280-285.

Luna-Martínez, J. E., Mejía-Terán, C. 2002. Brucellosis in Mexico: current status and trends. Veterinary Microbiology, 90, 19-30.

Mai, H. M., Irons, P. C., Kabir, J., Thompson, P. N., 2012. A large seroprevalence survey of brucellosis in cattle herds under diverse production systems in northern Nigeria. BMC Veterinary Research, 8(1), 144.

Makita, K., Fèvre, E. M., Waiswa, C., Eisler, M. C., Thrusfield, M., Welburn, S. C., 2011. Herd prevalence of bovine brucellosis and analysis of risk factors in cattle in urban and peri-urban areas of the Kampala economic zone, Uganda. BMC Veterinary Research, 7, 60.

Maleknejad, P., Peeri-DoGaheh, H., AmirZargar, A., Jafari, S., Fatollahzadeh, B., 2007. Diagnosis of brucellosis by use of BACTEC blood culture and confirmation by PCR. Veterinary Research, 62(4), 83-86.

Mamisashvili, E., Kracalik, I. T., Onashvili, T., Kerdzevadze, L., Goginashvili, K., Tigilauri, T., Donduashvili, M., Nikolaishvili, M., Beradze, I., Zakareishvili, M., Kokhreidze, M., Gelashvili, M., Vepkhvadze, N., Rácz, S. E., Elzer, P. H., Nikolich, M. P., Blackburn J. K., 2013. Seroprevalence of brucellosis in livestock within three endemic regions of the country of Georgia. Preventive Veterinary Medicine, 110(3-4), 554-557.

Mangalgi, S. S., Sajjan, A. G., Mohite S. T., 2012. Brucellosis*:* A cause for pyrexia of unknown origin. International Journal of Biological and Medical Research, 3(3), 2054-2058.

Mangalgi, S., and Sajjan, A., 2014. Comparison of three blood culture techniques in the diagnosis of human brucellosis. Journal of Laboratory Physicians, 6(1), 14-17.

Mantur, B. G., Amarnath, S. K., Parande, A. M., Patil, G. A., Walvekar, R. R., Desai, A. S., Parande, M. V., Shinde, R. S., Chandrashekar, M. R., Patil, S. D., Shivaram, C., Salagare, R. C., 2011. Comparison of a novel immunocapture assay with standard serological methods in the diagnosis of brucellosis. Clinical Laboratory, 57(5-6),333-341.

Mantur, B. and Amarnath, S., 2008. Brucellosis in India- a review. Journal of Biosciences, 33 (4), 539-547.

Mantur, B. G., 1988. Prevalence of brucellosis in north Karnataka- a serological and cultural study, MD Thesis, Karnataka University, Dharwad.

Mantur, B. G., Akki, A. S., Mangalgi, S. S., Patil, S. V., Gobbur, R. H., Peerapur, B. V., 2004. Childhood brucellosis--a microbiological, epidemiological and clinical study. Journal of Tropical Pediatrics, 50(3), 153-157.

Mantur, B. G., Bidari, L. H., Akki, A. S., Mulimani, M. S., Tikare, N. V., 2007. Diagnostic yield of blood clot culture in the accurate diagnosis of enteric fever and human brucellosis. Clinical Laboratory, 53(1-2), 57-61.

Mantur, B. G., Biradar, M. S., Bidri, R. C., Mulimani, M. S., Veerappa, Kariholu, P., Patil, S. B., Mangalgi, S. S., 2006. Protean clinical manifestations and diagnostic challenges of human brucellosis in adults: 16 years' experience in an endemic area. Journal of Medical Microbiology, 55(Pt 7), 897-903.

Mantur, B., Parande, A., Amarnath, S., Patil, G., Walvekar, R., Desai, A., Parande, M., Shinde, R., Chandrashekar, M., Patil, S., 2010. ELISA versus conventional methods of diagnosing endemic brucellosis. American Journal of Tropical Medicine and Hygiene, 83(2), 314-318.

Mantur, B.,andMangalgi, S., 2004. Evaluation of conventional Castaneda and lysis centrifugation blood culture techniques for diagnosis of human brucellosis. Journal of Clinical Microbiology, 42(9), 4327-4328.

Marianelli, C., Ciuchini, F., Tarantino, M., Pasquali, P., Adone, R., 2006. Molecular characterization of the rpoB gene in *Brucella* species: new potential molecular markers for genotyping. Microbes and Infection, 8(3), 860-865.

Marković-Denić, L., Trifunović, V. S., Zugić, V., Radojić, D., Stevanović, G., 2010. The first outbreak of brucellosis in the region of Sabac. Vojnosanitetski pregled, 67(8), 634-637.

Martínez de Tejada, G., Pizarro-Cerdá, J., Moreno, E., Moriyón, I., 1995. The outer membranes of *Brucella* spp. are resistant to bactericidal cationic peptides. Infection and Immunity, 63(8), 3054-3061.

Martin-Mazuelos, E., Nogales, M. C., Florez, C., Gómez-Mateos, J. M., Lozano, F., Sanchez, A., 1994. Outbreak of *Brucella melitensis* among microbiology laboratory workers. Journal of Clinical Microbiology, 32(8), 2035-2036.

Mathur, T. N., 1964. *Brucella* strains isolated from cows, buffaloes, goats, sheep and human beings at karnal: their significance with regard to the epidemiology of brucellosis. Indian Journal of Medical Research, 52, 1231-1240.

Mathur, T. N., 1968. The epidemiology of human brucellosis in Haryana with regard to 215 strains of *Brucella* isolated from man and animals. Indian Journal of Pathology and Bacteriology, 11, 244-249.

Matope, G., Bhebhe, E., Muma, J. B., Lund, A., Skjerve, E. 2011. Risk factors for *Brucella* spp. infection in smallholder household herds. Epidemiology and infection, 139(01), 157-164.

Matope, G., Bhebhe, E., Muma, J. B., Oloya, J., Madekurozwa, R. L., Lund, A., Skjerve, E., 2011. Seroprevalence of brucellosis and its associated risk factors in cattle from smallholder dairy farms in Zimbabwe. Tropical Animal Health Production, 43(5), 975-982.

Matope, G., Bhebhe, E., Muma, J. B., Skjerve, E., & Djønne, B., 2009. Characterization of some *Brucella* species from Zimbabwe by biochemical profiling and AMOS-PCR. BMC Research Notes, 2(1), 261.

Maurice, N. A., Wungak, S. Y., Gana, B. A., Nanven, M. B., Ngbede, E. O., Ibrahim, A., Aworh, M. K., Konzing, L., Hambolu, S. E. and Gugong, V. T., 2013. Seroprevalence of bovine brucellosis in northern Plateau State, North Central Nigeria. Asian Pacific Journal of Tropical Disease, 3(5), 337-340.

Mayer-Scholl, A., Draeger, A., Göllner, C., Scholz, H. C., Nöckler, K., 2010. Advancement of a multiplex PCR for the differentiation of all currently described *Brucella* species. Journal of Microbiological Methods, 80(1), 112-114.

McCaughey, W. J., Purcell, D. A., 1973. Brucellosis in bulls. Veterinary Record, 93(12), 336-337.

McGiven, J. A., Tucker, J. D., Perrett, L. L., Stack, J. A., Brew, S. D., MacMillan, A. P., 2003. Validation of FPA and cELISA for the detection of antibodies to *Brucella abortus* in cattle sera and comparison to SAT, CFT and iELISA. Journal of Immunological Methods, 278(1-2), 171-178.

McLean, D. R., Russell, N., Khan, M. Y., 1992. Neurobrucellosis: clinical and therapeutic features. Clinical Infectious Diseases, 15, 582-590.

Meeusen, E. N., Walker, J., Peters, A., Pastoret, P. P.,Jungersen, G., 2007. Current status of veterinary vaccines. Clinical microbiology reviews ,20(3), 489-510.

Megersa, B., Biffa, D., Niguse, F., Rufael, T., Asmare, K., Skjerve, E., 2011. Cattle brucellosis in traditional livestock husbandry practice in Southern and Eastern Ethiopia, and its zoonotic implication. Acta Veterinaria Scandinavica, 53(1), 24.

Mekonnen, H., Kalayou, S., Kyule, M., 2010. Serological survey of bovine brucellosis in Barka and Arado breeds (*Bos indicus*) of Western Tigray, Ethiopia. Preventive Veterinary Medicine, 94, 28-35.

Meltzer, E., Sidi, Y., Smolen, G., Banai, M., Bardenstein, S., Schwartz, E., 2010. Sexually transmitted brucellosis in humans. Clinical Infectious Diseases, 51(2), e12-15.

Memish, Z. A., Balkhy, H. H., 2004. Brucellosis and international travel. Journal of Travel Medicine, 11(1), 49-55.

Méndez Martínez, C., Páez Jiménez, A., Cortés-Blanco, M., Salmoral Chamizo, E., Mohedano Mohedano, E., Plata. C., Varo Baena, A., Martíinez Navarro, F., 2003. Brucellosis outbreak due to unpasteurized raw goat cheese in Andalucia (Spain), January March 2002. Eurosurveillance, 8(7), 164-168.

Mercier, E., Jumas-Bilak, E., Allardet-Servent, A., O'Callaghan, D., Ramuz, M., 1996. Polymorphism in *Brucella* strains detected by studying distribution of two short repetitive DNA elements. Journal of Clinical Microbiology, 34(5), 1299–1302.

Minharro, S., Silva, Mol, J. P., Dorneles, E. M. S., Pauletti, R. B., Neubauer, H., et al., 2013. Biotyping and genotyping (MLVA16) of *Brucella abortus* isolated from cattle in Brazil, 1977 to 2008. PLoS One, 8(12), e81152.

Mirnejad, R., Mohamadi, M., Piranfar, V., Mortazavi, S. M., Kachuei, R., 2013. A duplex PCR for rapid and simultaneous detection of *Brucella* spp. in human blood samples. Asian Pacific Journal of Tropical Medicine, 6(6), 453-456.

Mohammed, F. U., Ibrahim, S., Ajogi, I., Olaniyi, B. J., 2011. Prevalence of bovine brucellosis and risk factors assessment in cattle herds in Jigawa state, ISRN Veterinary Science, 2011 (2011), 132897.

Moreno, E., and Moriyón, I., 2006. The Genus *Brucella*. In The Prokaryotes (pp. 315-456). Springer New York.

Morgan, W. J., 1969. Brucellosis in animals: diagnosis and control. Proceedings of the Society for Experimental Biology and Medicine, 62(10), 1050-1052.

Mudaliar, S., Bhore, A., Pandit, D., 2003. Detection of antibodies to *Brucella abortus* in animal handlers. Indian Journal of Medical Science, 57(5), 181-186.

Muflihanah, H., Hatta, M., Rood, E., Scheelbeek, P., Abdoel, T. H., Smits, H. L., 2013. Brucellosis seroprevalence in Bali cattle with reproductive failure in South Sulawesi and *Brucella abortus* biovar 1 genotypes in the Eastern Indonesian archipelago. BMC Veterinary Researh, 9,233. doi: 10.1186/1746-6148-9-233.

Muhammad, N., Hossain, M. A., Musa, A. K., Mahmud, M. C., Paul, S. K., Rahman, M. A., Haque, N., Islam, M. T., Parvin. U. S., Khan. S. I., Nasreen. S. A., Mahmud. N. U., 2010. Seroprevalence of human brucellosis among the population at risk in rural area. Mymensingh Medical Journal, 19(1), 1-4.

Mukhtar, F., 2010. Brucellosis in a high risk occupational group: seroprevalence and analysis of risk factors. Journal of Pakistan Medical Association, 60(12), 1031-1034.

Mukhtar, F., Kokab, F., 2008. *Brucella* serology in abattoir workers. Journal of Ayub Medical College Abbottabad, 20(3), 57-61.

Muma, J. B., Godfroid, J., Samui, K. L., Skjerve, E., 2007. The role of *Brucella* infection in abortions among traditional cattle reared in proximity to wildlife on the Kafue flats of Zambia. Revue Scientifique Et Technique Office International des Epizooties, 26(3), 721-730.

Muma, J. B., Syakalima, M., Munyeme, M., Zulu, V. C., Simuunza, M., Kurata, M., 2013. Bovine tuberculosis and brucellosis in traditionally managed livestock in selected districts of Southern Province of Zambia. Veterinary Medicine International, 2013, 730367.

Mutnal, M. B., Purwar, S., Metgud, S. C., Nagmoti, M. B., Patil, C. S., 2007. PCR confirmation of cutaneous manifestation due to *Brucella melitensis.* Journal of Medical Microbiology, 56(Pt 2), 283-285.

Nagalingam, M., Shome, R., Balamurugan, V., Shome, B. R., Narayana Rao, K., Vivekananda, Isloor, S., Prabhudas, K., 2012. Molecular typing of *Brucella* species isolates from livestock and human. Tropical Animal Health Production, 44(1), 5-9.

Naha, K., Dasari, S., Pandit, V., Seshadri, S., 2012. A rare case of seronegative culture proven infection with *Brucella suis*, Australasian Medical Journal, 5(7), 340-343.

Nakouné, E., Debaere, O., Koumanda-Kotogne, F., Selekon, B., Samory, F., Talarmin, A., 2004. Serological surveillance of brucellosis and Q fever in cattle in the Central African Republic. Acta Tropica, 92(2), 147-51.

Nassonova, E. S., 2008. Pulsed field gel electrophoresis: Theory, instruments and application. Cell and Tissue Biology, 2(6), 557-565.

Navarro, E., Escribano, J., Fernández, J., Solera, J., 2002. Comparison of three different PCR methods for detection of *Brucella* spp. in human blood samples. FEMS Immunology and Medical Microbiology, 34(2), 147-151.

Navas, E., Guerrero, A., Cobo, J., Loza, E., 1993. Faster isolation of *Brucella* spp. from blood by isolator compared with BACTEC NR. Diagnostic Microbiology and Infectious Diseases, 16(1), 79-81.

Nawathe, D. R., Bhagwat, S. S., 1984. Observations on brucellosis in Nagpur region, India. International Journal of Zoonoses, 11(2), 229-232

Nelson, K. E., Ruben, F. L., Andersen, B., 1975. An unusual outbreak of brucellosis. Archives of Internal Medicine, 135(5), 691-695.

Nicoletti, P., 1980. The epidemiology of bovine brucellosis. Advances in Veterinary Sciences and Comparative Medicine, 24, 69-98.

Nicoletti, P., 2010. Brucellosis: past, present and future. Prilozi, 31(1), 21-32.

Nicoletti, P., Muraschi, T. F., 1966. Bacteriologic evaluation of serologic test procedures for the diagnosis of brucellosis in problem cattle herds. American Journal of Veterinary Research, 27(118), 689-694.

Nielsen, K. H., Kelly, L., Gall, D., Nicoletti, P., Kelly, W., 1995. Improved competitive enzyme immunoassay for the diagnosis of bovine brucellosis. Veterinary Immunology and Immunopathology, 46(3-4), 285-291.

Nielsen, K., 2002. Diagnosis of brucellosis by serology. Veterinary Microbiology, 90, 447-459.

Nielsen, K., Gall, D., 2001. Fluorescence polarization assay for the diagnosis of brucellosis: a review. Journal of Immunoassay and Immunochemistry, 22(3), 183-201.

Nielsen, K., Heck, F., Wagner, G., Stiller, J., Rosenbaum, B., Pugh, R. Flores, E., 1984. Comparative assessment of antibody isotypes to *Brucella abortus* by primary and secondary binding assays, Preventive Veterinary Medicine, 2, 197-204.

Nielsen, K., Smith, P., Gall, D., Perez, B., Cosma, C., Mueller, P., Trottier, J., Cote, G., Boag, L., Bosse, J., 1996. Development and validation of an indirect enzyme immunoassay for detection of antibody to *Brucella abortus* in milk. Veterinary Microbiology, 52(1-2), 165-73.

Nimri, L. F., 2003. Diagnosis of recent and relapsed cases of human brucellosis by PCR assay. BMC Infectious Diseases, 3, 5, doi:10.1186/1471-2334-3-5

Nizeyimana, G., Mwiine, F. N., Ayebazibwe. C., 2013. Comparative *Brucella abortus* antibody prevalence in cattle under contrasting husbandry practices in Uganda. Journal of South African Veterinary Association, 84(1), E1-5.

O'Callaghan, D., Cazevieille, C., Allardet-Servent, A., Boschiroli, M. L., Bourg, G., Foulongne, V., Frutos, P., Kulakov, Y., Ramuz, M. 1999. A homologue of the *Agrobacterium tumefaciens* VirB and *Bordetella pertussis* Ptl type IV secretion systems is essential for intracellular survival of *Brucella suis*. Moleular Microbiology, 33(6), 1210-1220.

Ocampo-Sosa, A. A., Agüero-Balbín, J., García-Lobo, J. M., 2005. Development of a new PCR assay to identify *Brucella abortus* biovars 5, 6 and 9 and the new subgroup 3b of biovar 3. Veterinary Microbiology, 110(1-2), 41-51.

O'Grady, D., Byrne, W., Kelleher, P., O'Callaghan, H., Kenny, K., Heneghan, T., Power, S., Egan, J., Ryan, F., 2014. A comparative assessment of culture and serology in the diagnosis of brucellosis in dairy cattle. The Veterinary Journal, 199(3), 370-375.

OIE (The World Organisation for Animal Health), 2009. Terrestrial animal health code; Chapter 2.4.3 Bovine brucellosis. Available from: http://web.oie.int/eng/normes/MMANUAL/2008/pdf/2.04.03_BOVINE_BRUCELL.pdf

OIE (The World Organisation for Animal Health), 2009. Terrestrial animal health code; Chapter 2.8.5 Porcine brucellosis, Available from: http://www.oie.int/fileadmin/Home/fr/Health_standards/tahm/2.08.05_PORCINE_BRUC.pdf

OIE (The World Organisation for Animal Health), 2010. Terrestrial animal health code; chapter 14.1. Caprine and ovine brucellosis (excluding *Brucella Ovis*).Availablefrom:http://web.oie.int/eng/normes/mcode/en_chapitre_1.14.1.pdf

O'Leary, S., Sheahan M, Sweeney T. 2006. *Brucella abortus* detection by PCR assay in blood, milk and lymph tissue of serologically positive cows. Research in Veterinary Science, 81(2), 170-176.

Omer, M. M., Musa, M. T., Bakhiet, M. R., Perrett, L., 2010. Brucellosis in camels, cattle and humans: associations and evaluation of serological tests used for diagnosis of the disease in certain nomadic localities in Sudan. Revue Scientifique Et Technique Office International des Epizooties, 29(3), 663-669.

Osterman, B. and Moriyón, I., 2006. International Committee on Systematics of Prokaryotes; Subcommittee on the taxonomy of *Brucella.* Minutes of the meeting, 17 September 2003, Pamplona, Spain. International Journal of Systematic and Evolutionary Microbiology, 56 (5), 1173-1175.

Ouahrani-Bettache, S., Soubrier, M., Liautard, J., 1996. IS6501-anchored PCR for the detection and identification of *Brucella* species and strains. Journal of Applied Bacteriology, 81(2), 154-160.

Ozturk, R., Mert, A., Kocak, F., Ozaras, R., Koksal, F., Tabak, F., Bilir, M., Aktuglu, Y., 2002. The diagnosis of brucellosis by use of BACTEC 9240 blood culture system. Diagnostic Microbiology and Infectious Diseases, 44(2), 133-135.

Paixão, T. A., Roux, C. M., den Hartigh, A. B., Sankaran-Walters, S., Dandekar, S., Santos, R. L., Tsolis, R. M., 2009. Establishment of systemic *Brucella melitensis* infection through the digestive tract requires urease, the type IV secretion system, and lipopolysaccharide O antigen. Infection and Immunity, 77(10), 4197-4208.

Palanduz, A., Palanduz, S., Güler, K., Güler, N. 2000. Brucellosis in a mother and her young infant: probable transmission by breast milk. International Journal of Infectious Diseases, 4(1), 55-56.

Panjarathinam R., 1984. Anti-brucella agglutinins in aborted women. Journal de Gynécologie Obstétrique et Biologie de la Reproduction (Paris), 13(4), 433-436.

Pappas, G., Akritidis, N., Bosilkovski, M., Tsianos, E., 2005. Brucellosis. New England Journal of Medicine, 352(22), 2325-2336.

Pappas, G., Papadimitriou, P., Akritidis, N., Christou, L., Tsianos, E., 2006. The new global map of human brucellosis. Lancet Infectious Diseases, 6(2), 91- 99.

Park, M. Y., Lee, C. S., Choi, Y. S., Park. S. J., Lee, J. S., Lee. H. B. 2005. A sporadic outbreak of human brucellosis in Korea. Journal of Korean Medical Science, 20(6), 941-946.

Parlak, M., Güdücüoğlu, H., Bayram, Y., Çıkman, A., Aypak, C., Kılıç, S., Berktaş, M., 2013. Identification and determination of antibiotic susceptibilities of *Brucella* strains isolated from patients in van, Turkey by conventional and molecular methods. International Journal of Medical Sciences, 10(10), 1406-1411.

Patel, M. D., Patel, P. R., Prajapati, M. G., Kanani, A. N., Tyagi, K. K., Fulsoundar, A. B., 2014. Prevalence and risk factor's analysis of bovine brucellosis in peri-urban areas under intensive system of production in Gujarat, India, Veterinary World, 7(7), 509-516.

Patil, N. B., Damle, A. S., Bhakare, J. B., Iravane, J. A., Khaparkhuntikar. M. N., Gajbhiye, P. S., 2013. Seropositivity for *Brucellosis* in veterinarians. Journal of Global Infectious Diseases, 5, 87-88.

Pawar, S. K., Ghorpade, M. V., Aundhkar, S., 2011. Culture positive *Brucella* endocarditis in a Case of Baloon Mitral Valvotomy. Online Journal of Health and Allied Sciences, 10(3), 12.

Pizarro-Cerdá, J., Desjardins, M., Moreno, E., Akira, S., Gorvel, J. P. 1999. Modulation of endocytosis in nuclear factor IL-6(-/-) macrophages is responsible for a high susceptibility to intracellular bacterial infection. The Journal of Immunology, 162(6), 3519-3526.

Pizarro-Cerdá, J., Méresse, S., Parton, R. G., van der Goot, G., Sola-Landa, A., López-Goñi, I., Moreno, E., Gorvel, J. P., 1998. *Brucella abortus* transits through the autophagic pathway and replicates in the endoplasmic reticulum of nonprofessional phagocytes. Infection and Immunity, 66(12), 5711-5724.

Pizarro-Cerdá, J., Moreno, E., Gorvel, J. P., 2000. Invasion and intracellular trafficking of *Brucella abortus* in nonphagocytic cells. Microbes and Infection, 2(7), 829-835.

Poester, F. P., Nielsen, K., Samartino, L. E., Yu, W. L., 2010. Diagnosis of brucellosis. Open Veterinary Science Journal, 4(1), 46-60.

Poole, P. M., Whitehouse, D. B., Gilchrist, M. M., 1972. A case of abortion consequent upon infection with *Brucella abortus* biotype 2. Journal of Clinical Pathology, 25(10), 882-884.

Porte, F., Naroeni, A., Ouahrani-Bettache, S., Liautard, J. P., 2003. Role of the *Brucella suis* lipopolysaccharide O antigen in phagosomal genesis and in inhibition of

phagosome-lysosome fusion in murine macrophages. Infection and Immunity, 71(3), 1481-1490.

Poulsen, K. P., Hutchins, F. T., McNulty. C. M., Tremblay, M., Zabala, C., Barragan, V., Lopez, L., Trueba, G., Bethel, J. W., 2014. Brucellosis in dairy cattle and goats in northern Ecuador. American Journal of Tropical Medicine and Hygiene, 90(4), 712-715.

Prakash, P., Bhansali, S., Gupta, E., Kothari, D., Mathur, A., Ambuwani, S. 2012. Epidemiology of brucellosis in high risk group & PUO patients of Western–Rajasthan. National Journal of Community Medicine, 3(1), 61-65.

Priyantha, M. A. R., 2011. Identification of biovars of *Brucella abortus* in aborted cattle and buffaloes herd in Sri Lanka. Veterinary World, 4(12), 542-545.

Queipo-Ortuño, M. I., Colmenero, J. D., Bravo, M. J., García-Ordoñez, M. A., Morata, P., 2008. Usefulness of a quantitative real-time PCR assay using serum samples to discriminate between inactive, serologically positive and active human brucellosis. Clinical Microbiology and Infection, 14(12), 1128-1134.

Queipo-Ortuρo, M. I., Colmenero, J. D., Baeza, G., Morata, P., 2005. Comparision between light cycler real time polymerase chain reaction (PCR) assay with serum and PCR, enzyme linked immunosorbant assay with whole blood samples for the diagnosis of human brucellosis. Clinical Infectious Diseases, 40(2), 260-264.

Radostits, O. M., Gay, C. C., Hinchcliff, K. W., Constable, P. D., 2007. Veterinary Medicine: A Textbook of the Diseases of Cattle, Horses, Sheep, Pigs and Goats. 10th edition. Saunders USA. pp. 963-984.

Rahman, A. K., Dirk, B., Fretin, D., Saegerman, C., Ahmed, M. U., Muhammad, N., Hossain A, Abatih E., 2012. Seroprevalence and risk factors for brucellosis in a high-risk group of individuals in Bangladesh. Foodborne Pathogens and Diseases, 9(3), 190-197.

Rahman, M. S., Faruk, M. O., Her, M., Kim, J. Y., Kang, S. I., Jung, S. C., 2011. Prevalence of brucellosis in ruminants in Bangladesh. Veterinarni Medicina, 56(8), 379-385.

Ramos, J. M., Bernal, E., Esguevillas, T., Lopez-Garcia, P., Gaztambide, M. S., Gutierrez, F., 2008. Non-imported brucellosis outbreak from unpasteurized raw milk in Moroccan immigrants in Spain. Epidemiology and Infection, 136(11), 1552-1555.

Rana, U. V., Sehgal, S., Bhardwaj, M. A. 1985. Sero-epidemiological study of brucellosis among workers of veterinary hospitals and slaughter house of Union Territory of Delhi. International Journal of Zoonoses, 12(1), 74-79.

Rankin, J. E., 1965. *Brucella abortus* in bulls: a study of twelve naturally-infected cases. Veterinary Record, 77, 132-135.

Reddy, R., Prejit, S. B., Vinod, V. K., Asha, K., 2014. Seroprevalence of brucellosis in slaughter cattle of Kerala, India. Journal of Foodborne and Zoonotic Diseases, 2(2), 27-29.

Renukaradhya, G. J., Isloor, S. and Rajasekhar M. 2002. Epidemiology, zoonotic aspects, vaccination and control/eradication of brucellosis in India. Veterinary Microbiology, 90(1-4), 183-195.

Rice, C. E., Boyes, B., 1971. Serum immunoglobulins in bovine brucellosis. New Zealand Veterinary Journal, 19(7), 146-154.

Ridler, A. L., Leyland, M. J., Fenwick, S. G., West, D. M., 2005. Demonstration of polymorphism among *Brucella ovis* field isolates by pulsed-field gel electrophoresis. Veterinary Microbiology, 108(1-2), 69-74.

Rijpens, N. P., Jannes, G., Van Asbroeck, M., Rossau, R., Herman, L. M.,1996. Direct detection of *Brucella* spp. in raw milk by PCR and reverse hybridization with 16S-23S rRNA spacer probes. Applied Environmental Microbiology. 1996, 62(5), 1683-1688.

Robinson, A. 2003. Guidelines for coordinated human and animal brucellosis surveillance In FAO Animal Production and Health Paper, pp156.

Rodrigues, A. L., Silva, S. K., Pinto, B. L., Silva, J. B., Tupinambás, U., 2013. Outbreak of laboratory-acquired *Brucella abortus* in Brazil: a case report. Revista da Sociedade Brasileira de Medicina Tropical, 46(6):791-794.

Román, K., Castillo, R., Gilman, R. H., Calderón, M., Vivar, A., Céspedes, M., Smits, H. L., Meléndez, P., Gotuzzo, E., Guerra, H., Maves, R. C., Matthias, M. A., Vinetz, J. M., Saito, M., 2013. A food borne outbreak of brucellosis at a police station cafeteria, Lima, Peru. American Journal of Tropical Medicine and Hygiene, 88(3), 552-558.

Romero, C., López-Goñi, I., 1999. Improved method for purification of bacterial DNA from bovine milk for detection of *Brucella* spp. by PCR. Applied Environmental Microbiology, 65(8), 3735-3737.

Romero, C., Pardo, M., Grillo, M. J., Diaz, R., Blasco, J. M., López -Goñi, I., 1995. Evaluation of PCR and indirect enzyme-linked immunosorbent assay on milk samples for diagnosis of brucellosis in dairy cattle. Journal of Clinical Microbiology, 33(12), 3198-3200.

Ron-Román, J., Ron-Garrido, L., Abatih, E., Celi-Erazo, M., Vizcaíno-Ordóñez, L., Calva-Pacheco, J., González-Andrade, P., Berkvens, D., Benítez-Ortíz, W., Brandt, J., Fretin, D., Saegerman, C., 2014. Human brucellosis in northwest Ecuador: typifying *Brucella* spp., seroprevalence, and associated risk factors. Vector Borne Zoonotic Diseases, 14(2), 124-133.

Rothman, K. J., and Greenland, S., Editors (1998). Modern Epidemiology, 2nd edition. Philadelphia: Lippincott-Raven.

Rubio, M., Barrio, B., Dı´az, R., 2001. Usefulness of Rose Bengal, Coombs and counter-immunoelectrophoresis for the diagnosis of human brucellosis cases with negative seroagglutination. Enfermedades Infecciosasy Microbiología Clínica, 19(8), 406-407.

Ruiz, J., Lorente, I., Pérez, J., Simarro, E., Martinez-Campos, L., 1997. Diagnosis of brucellosis by using blood cultures. Journal of Clinical Microbiology, 35(9), 2417-2418.

Ruiz-Castaneda, M. R., 1947. A practical method for routine blood cultures in brucellosis. Proceedings of Society for Experimental Biology and Medicine, 64(1), 114.

Rujeni, N., Mbanzamihigo, L., 2014. Prevalence of brucellosis among women Presenting with abortion/stillbirth in Huye, Rwanda. Journal of Tropical Medicine, 2014,740479.

Sahin, M., Genç, O., Unver, A., Otlu, S., 2008. Investigation of bovine brucellosis in the Northeastern Turkey. Tropical Animal Health Production, 40(4), 281-286.

Saini, S. S., Sharma, J. K., Kwatra, M. S., 1992. Assessment of some management factors responsible for prevalence of brucellosis among traditionally managed animal population of Punjab. Indian Journal of Animal Sciences, 62, 832-834.

Salari, M. H., Khalili, M. B., Hassanpour, G. R., 2003. Selected epidemiological features of human brucellosis in Yazd, Islamic Republic of Iran : 1993-1998. East Mediterranean Health Journal, 9(5-6), 1054-1060.

Saltoglu, N., Tasova, Y., Midikli, D., Aksu, H. S., Sanli, A., Dündar, I. H., 2004. Fever of unknown origin in Turkey: evaluation of 87 cases during a nine-year-period of study. Journal of Infection, 48(1), 81-85.

Samartino, L., Gregoret, R., Gall, D., Nielsen, K.. 1999. Fluorescence polarization assay: application to the diagnosis of bovine brucellosis in Argentina. Journal of Immunoassay. 20(3), 115-126.

Sangari, F. J., Agüero, J., 1994. Identification of *Brucella abortus* B19 vaccine strain by the detection of DNA polymorphism at the *ery* locus. Vaccine, 2(5), 435-438.

Sangari, F. J., Seoane, A., Rodríguez, M. C., Agüero, J., García Lobo, J. M., 2007. Characterization of the urease operon of Brucella abortus and ssessment of its role in virulence of the bacterium. Infection and Immunity, 75(2), 774-780.

Sanogo, M., Abatih, E., Thys, E., Fretin, D., Berkvens, D., Saegerman, C., 2012. Risk factors associated with brucellosis seropositivity among cattle in the central savannah-forest area of Ivory Coast. Preventive Veterinary Medicine, 107(1-2), 51-56.

Sathyanarayanan, V., Razak, A., Saravu, K., Ananthakrishna, S. B., Mukhyprana Prabhu, M., Vandana, K. E., 2011. Clinical profile of brucellosis from a tertiary care center in southern India. Asian Pacific Journal of Tropical Medicine, 4(5), 397-400.

Sayan, M., Erdenlig, S., Stack, J., Kilic, S., Guducuoglu, H., Aksoy, Y., Baklan, A., Etiler, N., 2011. A serological diagnostic survey for Brucella canis infection in Turkish patients with Brucellosis-like symptoms. Japanese Journal of Infectious Disease, 64(6), 516-519.

Scacchia, M., Di Provvido, A., Ippoliti, C., Kefle, U., Sebhatu, T. T., D'Angelo, A., De Massis, F., 2013. Prevalence of brucellosis in dairy cattle from the main dairy farming regions of Eritrea. Onderstepoort Journal of Veterinary Research, 80(1), 448.

Schelling, E., Diguimbaye, C., Daou, S., Nicolet, J., Boerlin, P., Tanner, M., Zinsstag, J., 2003. Brucellosis and Q-fever seroprevalences of nomadic pastoralists and their livestock in Chad. Preventive Veterinary Medicine, 61(4), 279-293.

Scholz, H. C. and Vergnaud, G., 2013. Molecular characterisation of *Brucella* species. Revue Scientifique Et Technique, Office International des Epizooties, 32(1), 149-162.

Scholz, H. C., Al Dahouk, S., Neubauer, H., Witte, M., et al., 2008b. Genetic diversity and phylogenetic relationships of bacteria belonging to the Ochrobactrum-*Brucella* group by recA and 16S rRNA gene-based comparative sequence analysis. Systimatic and Applied Microbiology, 31(1), 1-16.

Scholz, H. C., Hofer, E., Vergnaud, G., Le Fleche, P., Whatmore, A. M., Al Dahouk, S., Pfeffer, M., Krüger, M., Cloeckaert, A., Tomaso, H., 2009. Isolation of *Brucella microti* from mandibular lymph nodes of red foxes, *Vulpes vulpes*, in lower Austria. Vector Borne Zoonotic Diseases, 9(2), 153-156.

Scholz, H. C., Hubálek, Z., Sedlácek, I., Vergnaud, G., et al., 2008a. *Brucella microti* sp. nov., isolated from the common vole *Microtus arvalis*. International Journal of Systematic and Evolutionary Microbiology, 58 (Pt.2), 375-382.

Scholz, H. C., Nöckler, K., Göllner, C., Bahn, P., Vergnaud, G., Tomaso, H., Al Dahouk, S., Kämpfer, P., Cloeckaert, A., Maquart, M., Zygmunt, M. S., Whatmore A. M., Pfeffer, M., Huber, B., Busse, H. J., De, B. K., 2010. *Brucella inopinata* sp. nov., isolated from a breast implant infection. International Journal of Systematic and Evolutionary Microbiology, 60(Pt 4), 801-808.

Scott, J. C., Koylass, M. S., Stubberfield, M. R., Whatmore, A. M., 2007. Multiplex assay based on single-nucleotide polymorphisms for rapid identification of *Brucella* isolates at the species level. Applied Environmental Microbiology, 73(22), 7331-7337.

Seleem, M., Boyle, S. and Sriranganathan N., 2010. Brucellosis: a re-emerging zoonosis. Veterinary Microbiology, 140(3-4), 392-398.

Sen, M. R., Shukla, B. N., Goyal, R. K., 2002. Seroprevalence of brucellosis in and around Varanasi. Journal of Communicable Diseases, 34, 226-227.

Serpe, L., Gallo, P., Fidanza, N., Scaramuzzo, A., Fenizia, D., 1999. Single-step method for rapid detection of *Brucella* spp. in soft cheese by gene-specific polymerase chain reaction. Journal of Dairy Research, 66(2), 313-317.

Shabbir, M. Z., Nazir, M. M., Maqbool, A., Lateef, M., Shabbir, M. A., Ahmad, A., Rabbani, M., Yaqub, T., Sohail, M. U., Ijaz, M., 2011. Seroprevalence of *Neospora caninum* and *Brucella abortus* in dairy cattle herds with high abortion rates. Journal of Parasitology, 97(4), 740-742.

Shalmali, Panda, A. K., Chahota, R., 2012. Sero-prevalence of brucellosis in occupationally exposed human beings of Himachal Pradesh (India). Journal of Communicable Diseases, 44(2), 91-95.

Sharma, V. D., Sethi, M. S., Yadav, M. P., Dube, D. C., 1979. Sero-epidemiologic investigations on brucellosis in the states of Uttar Pradesh (U.P.) and Delhi (India). International Journal of Zoonoses, 6(2), 75-81.

Shimol, S. B., Dukhan, L., Belmaker, I., Bardenstein, S., Sibirsky, D., Barrett, C., Greenberg, D., 2012. Human brucellosis outbreak acquired through camel milk ingestion in southern Israel. Israel Medical Association Journal,14(8), 475-478.

Shome, R., Padmashree, B. S., Krithiga, N., Triveni, K., Sahay, S., Shome, B. R., Singh P., Rahman, H. 2014. Bovine Brucellosis in organized farms of India- An assessment of diagnostic assays and risk factors. Advances in Animal and Veterinary Sciences, 2 (10), 557-564.

Silva, I., Dangolla, A., Kulachelvy, K., 2000. Seroepidemiology of *Brucella abortus* infection in bovids in Sri Lanka. Preventive Veterinary Medicine, 46(1), 51-59.

Smirnova E. A., Vasin, N., Sandybaev, S., Klotchenko, M., Plotnikova, O., Chervyakova, A., Sansyzbay, and Kiselev, O., 2013. Current methods of human and animal brucellosis diagnostics. Advances in Infectious Diseases, 3(3), 177-184.

Smits, H. and Kadri, S., 2005. Brucellosis in India: a deceptive infectious disease. Indian Journal of Medical Research, 122 (5), 375-384.

Sola, M. C., da Veiga Jardim, E. A., de Freitas, M. R., de Mesquita, A. J., 2014. Real-time PCR detection of *Brucella* spp. DNA in lesions and viscera of bovine carcasses. Journal of Microbiological Methods, 104, 87-91.

Somily, A. M., Kambal, A. M., Naeem, T., Babay, H. A., Al Hedaithy, M. A., Al Anazi, A. R., Barry, M. A., Al Aska, A. I., Morshed, M. G., Murray, T. S., 2013. *Brucella* spp. isolated from respiratory sample and grown in *Mycobacterium* growth indicator tube (MGIT). Annals of Saudi Medicine, 33(4), 394-396.

Spera, J. M., Herrmann, C. K., Roset, M. S., Comerci, D. J., Ugalde, J. E., 2013. A *Brucella* virulence factor targets macrophages to trigger B-cell proliferation. Journal of Biological Chemistry, 288(28), 20208-202016.

Spera, J. M., Ugalde, J. E., Mucci, J., Comerci, D. J., Ugalde, R. A., 2006. A B lymphocyte mitogen is a *Brucella abortus* virulence factor required for persistent infection. Proceedings of National Academy of Science U. S. A., 103(44), 16514-16519.

Spickler, A. R., 2009. Ovine Epididymitis: *Brucella ovis*. Last Updated: July 2009, At http://www.cfsph.iastate.edu/DiseaseInfo/factsheets.php

Spickler, A. R., 2009. Porcine and Rangiferine brucellosis *Brucella suis*. Last Update July 2009, At http://www.cfsph.iastate.edu/DiseaseInfo/factsheets.php

Starr, T., Ng, T. W., Wehrly, T. D., Knodler, L. A., Celli, J., 2008. *Brucella* intracellular replication requires trafficking through the late endosomal/lysosomal compartment. Traffic, 9(5), 678-94.

Staszkiewicz, J., Lewis, C. M., Colville, J., Zervos, M., Band, J., 1991. Outbreak of *Brucella melitensis* among microbiology laboratory workers in a community hospital. Journal of Clinical Microbiology, 29(2), 287-290.

Sukumar, K., Tamilselvan, P. and Dorairajan, N., 2012. Studies on infertility in cattle and buffaloes caused by *Brucella abortus*. Tamilnadu Journal of Veterinary and Animal Sciences, 8 (4), 235-237.

Sümer, H., Sümer, Z., Alim, A., Nur, N., Ozdemir, L., 2003. Seroprevalence of *Brucella* in an elderly population in mid-Anatolia, Turkey. Journal of Health, Population and Nutrition, 21(2), 158-161.

Sutra, L., Caffin, J. P., Dubray, G., 1986. Role of milk immunoglobulins in the *Brucella* milk ring test. Veterinary Microbiology, 12(4), 359-366.

Swai, E. and Schoonman, L., 2012. A survey of zoonotic diseases in trade cattle slaughtered at Tanga city abattoir: a cause of public health concern. Asian Pacific Journal of Tropical Biomedicine, 2(1), 55-60.

Swai, E. S. and Schoonman, L., 2010. The use of Rose Bengal Plate Test to asses cattle exposure to *Brucella* infection in traditional and smallholder dairy production systems of Tanga region of Tanzania. Veterinary Medicine International, 2010, 837950.

Swai, E. S., Schoonman, L., 2009. Human brucellosis: seroprevalence and risk factors related to high risk occupational groups in Tanga Municipality, Tanzania. Zoonoses and Public Health, 56(4), 183-187.

Swaminathan, B., Barrett, T. J., Hunter, S. B., Tauxe, R. V., 2001. The CDC PulseNet Task Force. PulseNet: The Molecular Subtyping Network for Foodborne Bacterial Disease Surveillance, United States. Emerging Infectious Diseases, http://dx.doi.org/10.3201/eid0703.017303

Sylla, S., Sidimé, Y., Sun, Y., Doumbouya, S., Cong, Y., 2014. Seroprevalence investigation of bovine brucellosis in Macenta and Yomou, Guinea. Tropical Animal Health Production, 46(7), 1185-1191.

Tanner, M., Inlameia, O., Michel, A., Maxlhuza, G., Pondja, A,, Fafetine, J., Macucule, B., Zacarias, M., Manguele, J., Moiane, I. C., Marranangumbe, A. S., Mulandane, F., Schönfeld, C., Moserm I,, van Heldenm P., Machadom, A., 2014. Bovine tuberculosis and brucellosis in vattle and African buffalo in the Limpopo National Park, Mozambique. Transboundary and Emerging Diseases, doi: 10.1111/tbed.12210.

Tantillo, G. M., Di Pinto, A., Buonavoglia, C., 2003. Detection of *Brucella* spp. in soft cheese by semi-nested polymerase chain reaction. Journal of Dairy Research, 70(2), 245-247.

Tantillo, G., Di Pinto, A., Vergara, A., Buonavoglia, C., 2001. Polymerase chain reaction for the direct detection of *Brucella* spp. in milk and cheese. Journal of Food Protection, 64(2), 164-167.

Tesfaye, G., Tsegaye, W., Chanie, M., Abinet, F., 2011. Seroprevalence and associated risk factors of bovine brucellosis in Addis Ababa dairy farms. Tropical Animal Health Production, 43(5), 1001-1005.

Thakur, S. D., Kumar, R., Thapliyal, D. C., 2002. Human brucellosis: review of an under-diagnosed animal transmitted disease. Journal of Communicable Diseases, 34(4), 287-301.

Thakur, S. D., Thapliyal, D. C., 2002. Seroprevalence of brucellosis in man. Journal of Communicable Diseases, 34(2), 106-109.

Thapar, M. K., Young, E. J., 1986. Urban outbreak of goat cheese brucellosis. Pediatric Infectious Diseases Journal, 5(6), 640-643.

Tikare, N. V., Mantur, B. G., Bidari, L. H., 2008. Brucellar meningitis in an infant-evidence for human breast milk transmission. Journal of Tropical Pediatrics, 54(4), 272-274.

Tiller, R. V., Gee, J. E., Frace, M. A., Taylor, T. K., Setubal, J. C., Hoffmaster, A. R., De, B. K., 2010a. Characterization of novel *Brucella* strains originating from wild native rodent species in North Queensland, Australia. Applied Environmental Microbiology, 76(17), 5837-5845.

Tiller, R. V., Gee, J. E., Lonsway, D. R., Gribble, S., Bell, S. C., Jennison, A. V., Bates, J., Coulter, C., Hoffmaster, A. R., De, B. K., 2010b. Identification of an unusual *Brucella* strain (BO2) from a lung biopsy in a 52 year-old patient with chronic destructive pneumonia. BMC Microbiology, doi: 10.1186/1471-2180-10-23.

Trangadia, B., Rana, S. K., Mukharjee, F. and Srinivasan, V. A., 2010. Prevalence of brucellosis and infectious bovine rhinotrachitis in organized dairy farms in India. Tropical Animal Health Production, 42(2), 203-207.

Trichard, C. J., Herr, S., Bastianello, S. S., Roux, D., 1982. Unilateral orchitis in a bull caused by *Brucella abortus* biotype1. Journal of South African Veterinary Association, 53(1), 60-62.

Tscherneva E, Rijpens N, Naydensky C, Herman L. 1996. Repetitive element sequence based polymerase chain reaction for typing of *Brucella* strains. Veteerinary Microbiology 51(1-2), 169-78.

Tscherneva, E., Rijpens, N., Jersek, B., Herman, L. M., 2000. Differentiation of *Brucella* species by random amplified polymorphic DNA analysis. Journal of Applied Microbiology, 88(1), 69-80.

Tschopp, R., Abera, B., Sourou, S. Y., Guerne-Bleich, E., Aseffa, A., Wubete, A., Zinsstag, J., Young, D., 2013. Bovine tuberculosis and brucellosis prevalence in cattle from selected milk cooperatives in Arsi zone, Oromia region, Ethiopia. BMC Veterinary Research, 9,163, doi: 10.1186/1746-6148-9-163.

Tsolia, M., Drakonaki, S., Messaritaki, A., Farmakakis, T., Kostaki, M., Tsapra, H., Karpathios, T., 2002. Clinical features, complications and treatment outcome of childhood brucellosis in central Greece. Journal of Infection, 44(4), 257-262.

Uddin, M. J., Sanyal, S. C., Mustafa, A. S., Mokaddas. E. M., Salama, A. L., Cherian, G., Mounjaeed, M., Shuhaiber, H., 1998. The role of aggressive medical therapy along with early surgical intervention in the cure of *Brucella* endocarditis. Annals of Thoracic and Cardiovascular Surgery, 4(4), 209-213.

Upadhyay, S. R., Singh, R., Chandra, D., Singh, K. P., Rathore, B. S., 2007. Seroprevalence of bovine brucellosis in Uttar Pradesh. Journal of Immunology and Immunopathology, 9(1and2), 58-60.

Vajramani, G. V., Nagmoti, M. B., Patil, C. S., 2005. Neurobrucellosis presenting as an intra-medullary spinal cord abscess. Annals of Clinical Microbiology and Antimicrobials, 4,14.

Vancelik, S., Guraksin, A., Ayyildiz, A., 2008. Seroprevalence of human brucellosis in rural endemic areas in eastern Turkey. Tropical Doctor, 38(1), 42-43.

Vemulapalli, R., McQuiston, J. R., Schurig, G. G., Sriranganathan, N., Halling, S. M., Boyle, S. M., 1999. Identification of an *IS711* element interrupting the *wboA* gene of *Brucella abortus* vaccine strain RB51 and a PCR assay to distinguish strain RB51

from other *Brucella* species and strains. Clinical and Diagnostic Laboratory Immunology, 6(5), 760-764.

Verger, J. M., Grimont, F., Grimont, P. A. D. Grayon, M., 1985. Brucella, a monospecific genus as shown by deoxyribonucleic acid hybridization. International Journal of Systematic Bacteriology, 35(3), 292-295.

Verma, S., Katoch, R. C, Sharma, M., Nigam, P., 2000. Abortions and infertility in livestock due to brucellosis in Himachal Pradesh, India. Veterinarski Arhiv, 70(2), 75-82.

Vizcaíno, N., Caro-Hernández, P., Cloeckaert, A., Fernández-Lago, L., 2004. DNA polymorphism in the omp25/omp31 family of *Brucella* spp.: identification of a 1.7-kb inversion in *Brucella cetaceae* and of a 15.1-kb genomic island, absent from *Brucella ovis*, related to the synthesis of smooth lipopolysaccharide. Microbes and Infection, 6(9), 821-834.

Wallach, J. C., Miguel, S. E., Baldi, P. C., Guarnera, E., Goldbaum, F. A., Fossati, C. A., 1994. Urban outbreak of a *Brucella melitensis* infection in an Argentine family: clinical and diagnostic aspects. FEMS Immunology and Medical Microbiology, 8(1), 49-56.

Wallach, J. C., Samartino, L. E., Efron, A., Baldi, P. C., 1997. Human infection by *Brucella melitensis*: an outbreak attributed to contact with infected goats. Immunology and Medical Microbiology, 19(4), 315-321.

Weinhäupl, I., Schöpf, K. C., Khaschabi, D., Kapaga, A. M., Msami, H. M., 2000. Investigations on the prevalence of bovine tuberculosis and brucellosis in dairy cattle in Dar es Salaam region and in zebu cattle in Lugoba area, Tanzania. Tropical Animal Health Production, 32(3), 147-154.

Whatmore, A. M., Davison, N., Cloeckaert, A., Al Dahouk, S., Zygmunt, M. S., Brew. S. D., Perrett, L. L., Koylass, M. S., Vergnaud, G., Quance, C., Scholz, H. C., Dick, E. J, Jr., Hubbard, G., Schlabritz-Loutsevitch, N. E., 2014. *Brucella papionis* sp. nov. isolated from baboons (*Papio* spp.). International Journal of Systematic and Evolutionary Microbiology, doi: 10.1099/ijs.0.065482-0.

Whatmore, A. M., Murphy, T. J., Shankster, S., Young, E., Cutler, S. J., Macmillan, A. P., 2005. Use of amplified fragment length polymorphism to identify and type *Brucella* isolates of medical and veterinary interest. Journal of Clinical Microbiology, 43(2), 761-769.

Whatmore, A. M., Perrett, L. L., MacMillan, A. P., 2007. Characterisation of the genetic diversity of *Brucella* by multilocus sequencing, BMC Microbiology, 7(1), 34.

White, P. G., Wilson, J. B., 1951. Differentiation of smooth and nonsmooth colonies of *Brucellae*. Journal of Bacteriology, 61(2), 239-240.

Woolhouse, M. E., Gowtage-Sequeria, S., 2005. Host range and emerging and reemerging pathogens. Emerging Infectious Diseases Journal, 11(12),1842-1847.

World Health Organization, 1959. Zoonoses: Second report of the joint WHO/FAO Expert Committee, WHO: Geneva, Switzerland.

Wright, A. E., Smith, F., 1897. On the application of the serum test to the differential diagnosis of typhoid and Malta fever. The Lancet, 1, 656-659.

Xavier, M. N., Paixão, T. A., Hartigh, A. B., Tsolis, R. M., Santos, R. L., 2010. Pathogenesis of *Brucella* spp. Open Veterinary Science Journal, 4(1), 109-118.

Yacoub, A. A., Bakr, S., Hameed, A. M., Al-Thamery, A. A., Fartoci, M. J., 2006. Seroepidemiology of selected zoonotic infections in Basra region of Iraq. East Mediterranean Health Journal, 12(1-2), 112-118.

Yagupsky, P., 1999. Detection of Brucellae in blood cultures. Journal of Clinical Microbiology, 37(11), 3437-3442.

Yagupsky, P., Baron, E. J., 2005. Laboratory exposures to Brucellae and implications for bioterrorism. Emerging Infectious Disease, 11(8), 1180-1185.

Yohannes, M. and Gill, J. P. S., 2011. Seroepidemiological survey of human brucellosis in and around Ludhiana, India. Emerging Health Threats Journal, 4, 10.3402/ehtj.v4i0.7361.

Young, E. J., 1983. Human brucellosis. Reviews of Infectious Diseases, 5(5), 821-842.

Young, E. J., 1995. An overview of human brucellosis. Clinical Infectious Diseases, 21(2), 283-289.

Zerva, L., Bourantas, K., Mitka, S., Kansouzidou, A., Legakis, N. J., 2001. Serum is the preferred clinical specimen for diagnosis of human brucellosis by PCR. Journal of Clinical Microbiology, 39(4), 1661-1664.

Zeytinoğlu, A., Turhan, A., Altuğlu, I., Bilgiç, A., Abdoel, T. H., Smits, H. L., 2006. Comparison of *Brucella* immunoglobulin M and G flow assays with serum agglutination and 2-mercaptoethanol tests in the diagnosis of brucellosis. Clinical Chemistry and Laboratory Medicine, 44(2), 180-184.

Zhang, B., Wear, D. J., Stojadinovic, A., Izadjoo, M., 2013. Sequential real-time PCR assays applied to identification of genomic signatures in formalin-fixed paraffin-embedded tissues: a case report about brucella-induced osteomyelitis. Military Medicine, 178(1), 88-94.

Zolzaya, B., Selenge, T., Narangarav, T., Gantsetseg, D., Erdenechimeg, D., Zinsstag, J., Schelling, E., 2014. Representative seroprevalences of human and livestock brucellosis in two Mongolian provinces. EcoHealth, 11(3), 356-371.

Zowghi, E., Ebadi, A., Mohseni, B., 1990. Isolation of *Brucella* organism from the milk of seronegative cows. Revue Scientifique et Technique Office International des Epizooties, 9 (4), 1175-1178.

Appendix

Media:-
Amies transport medium

Ingredients	Gms / Litre
Sodium chloride	3.000
Potassium chloride	0.200
Calcium chloride	0.100
Magnesium chloride	0.100
Monopotassium phosphate	0.200
Disodium phosphate	1.150
Sodium thioglycollate	1.000
Agar	4.000
Final pH (at 25°C) 7.3±0.2	

Suspend 9.75 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Dispense in screw cap bottles or tubes in 6 ml or desired quantity. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool in an upright position

Sheep Blood agar base

Ingredients	Gms / Litre
Casein enzymic hydrolysate	14.000
Peptic digest of animal tissue	4.500
Yeast extract	4.500
Sodium chloride	5.000
Agar	12.500
Final pH (at 25°C) 7.3±0.2	

Suspend 40.5 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15

minutes. Cool to 45-50°C and aseptically add 5% sterile sheep blood. Mix well and pour into sterile Petri plates.

***Brucella* agar base**

Ingredients	**Gms / Litre**
Casein enzymic hydrolysate	10.000
Yeast extract	2.000
Dextrose	1.000
Sodium chloride	5.000
Sodium bisulphite	0.100
Agar	15.000
Final pH (at 25°C) 7.0±0.2	

Suspend 21.55 grams in 500 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add sterile 5% v/v inactivated Horse Serum (inactivated by heating at 56°C for 30 minutes) and rehydrated contents of one vial of Brucella Selective Supplement .

Brucella Selective Supplement (Per vial sufficient for 500 ml medium)

Ingredients	**Concentration**
Polymyxin B sulphate	2500IU
Bacitracin	12500IU
Nystatin	50000IU
Cycloheximide	50mg

Naildixic acid	2.500mg
Vancomycin	10mg

Rehydrate the contents of 1 vial aseptically with 10 ml of 50% methanol. Shake to form a uniform suspension. Add the contents to 500 ml of sterile, molten, cooled (45-50ºC) media.

***Brucella* broth base**

Ingredients	**Gms / Litre**
Casein enzymic hydrolysate	10.000
Peptic digest of animal tissue	10.000
Yeast extract	2.000
Dextrose	1.000
Sodium chloride	5.000
Sodium bisulphite	0.100
Final pH (at 25°C) 7.0±0.2	

Suspend 14.05 grams in 500 ml distilled water. Heat if necessary to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Cool to 45-50°C and aseptically add sterile 5% v/v inactivated horse serum (Inactivate by heating at 56°C for 30 minutes) and add rehydrated contents of one vial of Brucella Selective Supplement. Mix well before pouring into sterile tubes.

MacConkey agar

Ingredients	Gms / Litre
Peptones (meat and casein)	3.000
Pancreatic digest of gelatin	17.000
Lactose monohydrate	10.000
Bile salts	1.500
Sodium chloride	5.000
Crystal violet	0.001
Final pH (at 25°C) 7.1±0.2	

Suspend 49.53 grams of dehydrated medium in 1000 ml purified/distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes i.e. validated cycle. Avoid overheating. Cool to 45-50°C. Mix well before pouring into sterile Petri plates. The surface of the medium should be dry when inoculated

Nitrate broth

Ingredients	Gms / Litre
Peptic digest of animal tissue	5.000
Meat extract	3.000
Potassium nitrate	1.000
Sodium chloride	30.000
Final pH (at 25°C) 7.0±0.2	

Suspend 39 grams in 1000 ml distilled water. Heat if necessaryto dissolve the medium completely. Dispense in tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

MR-VP Medium (Glucose Phosphate Broth)

Ingredients	**Gms / Litre**
Buffered peptone	7.000
Dextrose	5.000
Dipotassium phosphate	5.000
Final pH (at 25°C) 6.9±0.2	

Suspend 17 grams in 1000 ml of distilled water. Heat if necessary to dissolve the medium completely. Distribute in test tubes in 10 ml amounts and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

Urea Agar Base, Christensen

Ingredients	**Gms / Litre**
Peptic digest of animal tissue	1.000
Dextrose	1.000
Sodium chloride	5.000
Monopotassium phosphate	2.000
Phenol red	0.012
Agar	15.000
Final pH (at 25°C) 6.8±0.2	

Suspend 24.01 grams in 950 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 10 lbs pressure (115°C) for 20 minutes. Cool to 50°C and aseptically add 50 ml of sterile 40% Urea Solution and mix well. Dispense into sterile tubes and allow to set in a slanting position. Do not overheat or reheat the medium as urea decomposes very easily.

Brain Heart Infusion Agar

Ingredients	**Gms / Litre**
Calf brain, infusion from	200.000
Beef heart, infusion from	250.000
Proteose peptone	10.000
Dextrose	2.000
Sodium chloride	5.000
Disodium phosphate	2.500
Agar 15.000	

Suspend 52 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. Mix well before pouring.

Brain Heart Infusion broth

Ingredients	**Gms / Litre**
Calf brain, infusion from	200.000
Beef heart, infusion from	250.000
Proteose peptone	10.000

Dextrose	2.000
Sodium chloride	5.000
Disodium phosphate	2.500
Agar	15.000

Suspend 37 grams in 1000 ml distilled water. Heat if necessary to dissolve the medium completely.Dispense into bottles or tubes and sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. For best results, the medium should be used on the day it is prepared, otherwise, it should be boiled or steamed for a few minutes and then cooled before use.

Soyabean Casein Digest Agar (Tryptone Soya Agar)

Ingredients	**Gms / Litre**
Pancreatic digest of casein	15.000
Papaic digest of soyabean meal	5.000
Sodium chloride	5.000
Agar	15.000
Final pH (at 25°C) 7.3±0.2	

Suspend 40 grams in 1000 ml distilled water. Heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 15 minutes. If desired, aseptically add 5% v/v defibrinated blood in previously cooled medium to 45-50°C for cultivation. Mix well and pour into sterile Petri plates.

Diagnostic Kits:

- Protein G based indirect ELISA kit for diagnosis of bovine brucellosis developed by National Institute of Veterinary Epidemiology and Disease Informatics (NIVEDI, formerly PD_ADMAS), Hebbal, Bengaluru, India
- Nova Tec Immunodiagnostica GmbH IgG and IgM ELISA kit

Buffers and Reagents:

1. TRIS stock (1M)

Component	**Quantity**
TRIS	121.14
D/W	1000 ml

Adjust the pH to 8.0 if necessary

2. EDTA Stock

Component	**Quantity**
EDTA	372.24 g
D/W	1000 ml

Adjust the pH to 8.0 if necessary

3. TE Buffer (10 mM Tris:1 mM EDTA, pH 8.0):

Component	**Quantity**
Tris (1M, pH 8.0)	10 ml
EDTA (1M, pH 8.0)	1 ml
D/W	89 ml

Adjust the pH to 8.0 if necessary

4. 1% PFGE agarose in TE Buffer:

Component	**Quantity**

PFGE grade agarose	1 gm
TE buffer (pH 8.0)	10 ml

5. Cell lysis buffer (50mM Tris:50mM EDTA, pH 8.0 + 1% Sarcosyl)

Component	**Quantity**
Tris (1M, pH 8.0)	5 ml
EDTA (1M, pH 8.0)	5 ml
Sarcosyl	1 gm
D/W	90 ml

6. Cell Lysis/Proteinase K Buffer

Component	**Quantity**
Cell lysis buffer	5 ml
Proteinase K (20 mg/ml)	25 µl

7. Cell Suspension Buffer (100 mM Tris:100 mM EDTA, pH 8.0)

Component	**Quantity**
Tris (1M, pH 8.0)	1.211 g
EDTA (1M, pH 8.0)	3.72 g
D/W	100ml

Mix to dissolve and adjust pH to 8. Store this solution at room temperature

8. Preparation of phosphate-buffered saline (0.01 M; pH 7.2)

Component	**Quantity**
Na2HPO4 (anhydrous)	1.09 g
NaH2PO4 (anhydrous)	0.32 g
NaCl	9.0 g
D/W	1000 ml

Mix to dissolve and adjust pH to 7.2. Store this solution at room temperature.

Publications

Research Articles:-

- **Pathak AD**, Dubal, ZB, Doijad S, Raorane A, Rodrigues S, Naik R, Naik-Gaonkar S, Naik R, Barbuddhe SB (2014). Human brucellosis among pyrexia of unknown origin cases and occupationally exposed individuals in Goa Region, India. *Emerging Health Threats Journal*, 7, 23846, doi: 10.3402/ehtj.v7.23846.

- Raorane AV, Doijad S, Katkar S, **Pathak A**, Poharkar K, Dubal ZB, Barbuddhe SB (2014). Prevalence of *Listeria* spp. in animals and associated environment. *Advances in Animal and Veterinary Sciences*, 2 (2): 81-85.

- Raorane AV, Chothe S, Dubal ZB, Barbuddhe SB, Karunakaran M, Doijad S, **Pathak A,** Poharkar K, Singh NP. (2013). Antibiotic resistance of the pathogens isolated from bovine mastitis in Goa. *Ruminant Science*, 2(2):139-144.

- Raorane AV, Doijad SP, Poharkar KV, **Pathak A**, Bhosle S, Barbuddhe SB (2015). Isolation and genotypic characterization of *Listeria monocytogenes* from pork and pork products. *International Journal of Current Microbiology and Applied Sciences*, 4(1): 788-798.

Conference Participation

Poster presentation:-

- **Pathak A.**, Doijad S., Dubal Z. B. , Garg S., Raorane A., Barbuddhe S.B. Incidences of brucellosis among the cattle (with reproductive disorders) in Goa. Poster presented at 54th Annual Conference of Association of Microbiologist of India, (AMI, 2013) held during 17-20th November, 2013 at Maharshi Dayanand University, Rohtak, Haryana.
- Poharkar AG, **Pathak A.**, Doijad S. and Barbuddhe S. B., Need for "One health approach" and awareness for understanding about *Listeria monocytogenes.* Poster presented at "International Symposium on Problems of Listeriosis XVIII" (ISOPOL XVIII), Goa, India, 19-22nd September 2013.
- Raorane A**.,** Doijad S., **Pathak A**., Poharkar K., Dubal Z., Barbuddhe S. B., Isolation and characterization of *Listeria* from piggery environment and pork products. Poster presented at "International Symposium on Problems of Listeriosis XVIII" (ISOPOL XVIII), Goa, India, 19-22nd September 2013.
- Poharkar KV, Kerkar S., Doijad SP., Raorane AV**., Pathak A**., Singh NP., Barbuddhe SB. Prevalence of *Listeria* species from mangrove environment. Poster presented at "International Symposium on Problems of Listeriosis XVIII" (ISOPOL XVIII), Goa, India, 19-22nd September 2013.